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METABOLISM OF THE FURAN AND HYDROFURAN DERIVATIVES IN THE ANIMAL ORGANISM.

By NOBUYOSHI SUZUKI.

(*From the Laboratory of Medicine, Kyoto Imperial University, Kyoto.*)

(Received for publication, February 15, 1918.)

Production of Hydroxylmethylpyromucic Acid from Chitose.

Studies of the intermediary products of catabolism have made remarkable progress since Knoop[1] in 1904, employed aromatic fatty acids in his experiments. The aliphatic fatty acids, which are highly resistant to the action of most chemical reagents, are completely oxidized in the animal organism into their end-products, carbon dioxide and water. On the other hand, the benzene nucleus is strongly resistant to oxidation *in vivo*, and the fate of aromatic fatty acid can be followed in the animal organism. The furan nucleus is quite as refractory in the animal organisms as the benzene nucleus, and the furan compounds are in some respects closely related to natural substances, expecially carbohydrates.

Jaffé and Cohn,[2] giving furfurol to animals, found that this substance had been changed *in vivo* into furfuracrylic acid in a manner quite analogous to Perkins' synthesis, while on the other hand, it also gave pyromucic acid by oxidation. These were coupled with glycocoll and excreted as furfuracryluric acid and pyromycuric acid. Furthermore, Sasaki[3] observed that furfurpropionic acid is changed in the animal body into furfuracryluric acid and pyromycuric acid. From this fact it has been ascertained that the saturated fatty acids change into α-β-unsaturated acids. Oxymethylfurfurol[4] is also easily produced from cellulose or from saccharides.

[1] Knoop, F., Der Abbau aromatischer Fettsäuren im Tierkörper, Monograph, Freiburg, 1904; *Beit. chem. Physiol. u. Path.*, 1905, vi, 150.

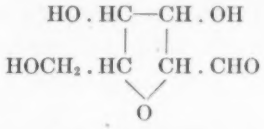
[2] Jaffé, M., and Cohn, R., *Ber. chem. Ges.*, 1887, xx, 2311.

[3] Sasaki, T., *Biochem. Z.*, 1910, xxv, 272.

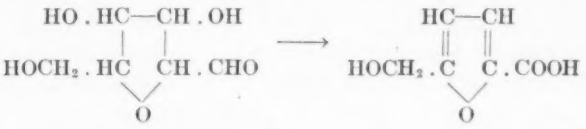
[4] Erdmann, E., *Ber. chem. Ges.*, 1910, xliii, 2391. Van Ekenstein, W. A., and Blanksma, J. J., *ibid.*, 2355.

S.D.B.

Chitose is prepared from glucosamine hydrochloride by Fischer and Tiemann's[5] method. Fischer and Andreae[6] recognized it as a hydrofuran derivative with the following constitution:

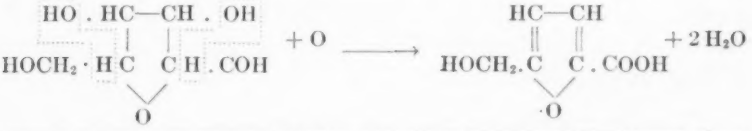


Thus glucosamine is often considered a derivative of chitose and called chitosamine. Though chitose is not properly to be regarded as a sugar, it resembles a monosaccharide in some respects. Under the direction of Professor Sasaki, I have studied the metabolism of chitose in the animal organism. I have been able to isolate from the urine of animals to which chitose had been given, a substance which crystallizes especially well, and I have identified it as hydroxylmethylpyromucic acid.



Instances are not wanting of aldehyde groups being oxidized to carboxylic acids in the animal organism, but the production of a furan compound, that is, the double linkage at α-β-position, is worthy of note.

Many hydroxyl acids change into unsaturated acids corresponding to them, *e.g.*, malic acid into fumaric acid, citric acid into aconic acid, and mucic acid into furfurandicarboxyl acid (dehydromucic acid[7]). The conversion of chitose into hydroxylmethylpyromucic acid can be represented as follows:



[5] Fischer, E., and Tiemann, F., *Ber. chem. Ges.*, 1894, xxvii, 139.

[6] Fischer, E., and Andreae, E., *Ber. chem. Ges.*, 1903, xxxvi, 2589.

[7] Seelig, E., *Ber. chem. Ges.*, 1879, xii, 1083. Fischer, E., *ibid.*, 1891, xxiv, 2140. Hill, H. B., *ibid.*, 1899, xxxii, 1221. Heinzelmann, R., *Ann. Chem.*, 1878, exciii, 184.

This result is in accord with the view that α-β-unsaturated acids are derived from hydroxyl acids in the body.

$$\begin{array}{l} \text{R . CH . CH . COOH} \longrightarrow \text{R . CH:CHCOOH} \\ \quad\;\; | \quad\; | \\ \quad\; \text{OH} \;\; \text{H} \end{array}$$

Thus Dakin[8] has isolated cinnamoylglycocoll by giving phenyl-β-oxypropionic acid to dogs.

The author is making further studies of chitonic acid, isosaccharic acid, and oxymethylfurfural, and expects to report them later.

EXPERIMENTAL.

Chitose cannot be crystallized, and since it cannot be prepared in pure form, the following experiment was first made. 15 gm. of chitose syrup were dissolved in water, acidified by addition of phosphoric acid, and extracted with ether using the Kumagawa-Suto liquid extractor for 6, 10, and 40 hours. Only a small quantity of syrup was obtained from each of the extracts, but no crystalline substance. Thus, the 15 gm. of chitose syrup used contained no crystalline furan compounds, and certainly no hydroxylmethylpyromucic acid.

Chitose was given to rabbits which had been fed with "okara"[9] by injecting its solution subcutaneously, or giving it by stomach tube. The urine of the animals was collected for 2 days after the chitose was given and evaporated to a thin syrup, which was extracted thoroughly with hot 90 per cent alcohol. The evaporated alcoholic extract was dissolved in water, acidified with phosphoric acid, and extracted with ether in the Kumagawa-Suto extracting apparatus. From the first (10 hours) and the second (15 hours) ether extracts, after the evaporation of ether, a beautifully crystalline substance was obtained. From the third (40 hours) and the fourth (20 hours) fractions, a syrup was obtained, but no crystalline substance could be isolated. The crystalline substances from the first and second extracts were purified on a

[8] Dakin, H. D., *J. Biol. Chem.*, 1909, vi, 213.

[9] Refuse of beans produced in the manufacture of "tofu" which is made from beans strained through rough cloth, after they have been boiled soft and ground into a semiliquid substance.

TABLE I.

Experiment No.	Sex.	Weight of rabbit.	Quantity of chitose.	Method.	Total quantity of urine.	Substance obtained	Per cent of substance obtained to that given.
		kg.	*gm.*		*cc.*	*gm.*	*per cent*
I	♀	2.56	10	Dissolved in 15 cc. water + 2 gm. sodium carbonate. *Per os.*	1st day, 360 2nd " 290	0.782	7.82
II	♀	2.22	15	Dissolved in 25 cc. water. *Per os.*	1st day, 100 2nd " 90	0.895	5.97
III	♀	2.25	15	Dissolved in 25 cc. water + 2 gm. Na_2CO_3. *Per os.*	1st day, 160 2nd " 120	0.983	6.55
IV	♂	2.55	8 Portion I, 3 Portion II, 5	I. Portion dissolved in 6 cc. water. II. Portion dissolved in 10 cc. water. Subcutaneously.	1st day, 250 2nd " 210 3rd " 180	0.572	7.15
V	♀	2.45	10 Portion I, 5 Portion II, 5	Dissolved in 10 cc. water. 1 gm. sodium carbonate. Subcutaneously.	1st day, 200 2nd " 185 3rd " 220	0.832	8.3
VI	♀	3.00	40 in four portions; 10 daily.	Dissolved in 20 cc. water. 2 gm. sodium carbonate. *Per os.*	1st day, 285 2nd " 220 3rd " 160 4th " 200 5th " 150	1.72	4.3
VII	♂	3.80	20 in four portions; 5 daily.	Dissolved in 10 cc. water. 1 gm. sodium carbonate. Subcutaneously.	1st day, 260 2nd " 225 3rd " 230 4th " 150 5th " 215	1.08	5.04

clay plate. The crystals were then recrystallized from boiling water after adding animal charcoal. The beautiful, fine, colorless, needle-shaped crystals were then dried in a vacuum desiccator over sulfuric acid and weighed. The results of seven experiments with animals are shown in Table I.

The percentages of the quantity of the crystals obtained from the urine of the animals to which chitose had been given, to the amounts of chitose given were from 4.3 to 8.32. The substance melted at 165–167° (uncorrected), turning brown and evolving gas. It dissolves readily in water, alcohol, and glacial acetic acid, but is difficultly soluble in ether, and almost wholly insoluble in benzene, chloroform, and carbon disulfide. For analysis it was again dissolved in water, to free it entirely from animal charcoal, and after addition of phosphoric acid, again extracted with ether. The crystals obtained were then recrystallized from water and dried over sulfuric acid in a vacuum desiccator; they were further dried at 100°, until their weight became constant.

0.1811 gm. substance gave 0.3346 gm. CO_2 and 0.0685 gm. H_2O.

	Calculated for $C_6H_8O_4$:	Found:
C	50.69	50.39
H	4.26	4.23

The properties of the acid were identical with those recorded by Hill and Jennings[10] and by Fischer and Andreae.[6]

[10] Hill, H. B., and Jennings, W. L., *Am. Chem. J.*, 1893, xv, 181.

BEHAVIOR OF CINNAMIC ACID AND ITS DERIVATIVES IN THE ANIMAL BODY.

By HIDEZO ANDO.

(From the Laboratory of Medicine, Kyoto Imperial University, Kyoto.)

(Received for publication, February 15, 1918.)

Magnus-Levy[1] has proved by experiment the fact that the α-amino-acids, which generally are readily burnt up in the animal organism, escape destruction if the amino group is benzoylated. At the suggestion of Professor Sasaki, I have tried to determine whether the unsaturated benzoylated amino-acids would remain unattacked in the animal body. Experiments were made on dogs and rabbits with benzoyl-α-aminocinnamic acid, with *p*-hydroxylbenzoyl-α-aminocinnamic acid, and with cinnamoyl tyrosine. It was found that benzoyl-α-aminocinnamic acid was almost quantitatively eliminated in the urine unchanged. On the other hand, *p*-hydroxylbenzoyl-α-aminocinnamic acid could be found in the urine after subcutaneous administration only, and in varying and reduced quantity. Only once an insignificant percentage was found in the urine after administration by mouth.

This difference as to the excretion of the two compounds of similar chemical constitution led us to try the same experiment with benzoyl-*o*-aminocinnamic acid. This also was excreted in the urine almost quantitatively after administration by mouth or subcutaneously. Cinnamoyl tyrosine, given to dogs, appeared to be completely oxidized, since no trace of it was found in the urine. In rabbits, however, after subcutaneous administration it could be traced in minimal quantities in the urine; when given by mouth, hippuric acid could be isolated from the urine, but no other derivative. Unlike benzoyl tyrosine, therefore, cinnamoyl tyrosine is further split up in the animal body.

[1] Magnus-Levy, A., *Biochem. Z.*, 1907, vi, 555.

Substance.	*Formula.*	*Fate.*
Benzoyl-α-amino-p-hydroxylcinnamic acid.	$C_6H_4(OH).CH : C(COOH).NH.CO.C_6H_5$	Chiefly decomposed.
Benzoyl-α-aminocinnamic acid.	$C_6H_5.CH : C(COOH).NH.CO.C_6H_5$	Excreted unchanged.
Benzoyl-o-aminocinnamic acid.	$C_6H_5.CO.NH.C_6H_4.CH : CH.COOH$	" "
Cinnamoyl tyrosine.	$OH.C_6H_4.CH_2.CH(COOH)NH.CO.CH : CH.C_6H_5$	Decomposed.

EXPERIMENTAL.

Benzoyl-α-aminocinnamic acid and p-hydroxylbenzoylaminocinnamic acid were prepared according to Erlenmeyer's[2] directions. Benzoyl-o-aminocinnamic acid was obtained synthetically by benzoylating o-aminocinnamic acid, and was found to contain the proper percentage of nitrogen.

The cinnamoyl-l-tyrosine has not been synthesized previously and was prepared as follows:

(a) 25 gm. of tyrosine ethyl ester hydrochloride were suspended in 250 cc. of chloroform, cooled to 0°C., and 103 cc. of normal sodium hydroxide were added. The tyrosine ester thus liberated went into the chloroform.

(b) 20 gm. of cinnamoyl chloride were dissolved in 125 cc. of anhydrous chloroform.

(c) Approximately 12 gm. of dry sodium carbonate were dissolved in 50 cc. of water. Half of the cinnamoyl chloride solution was added to the cooled tyrosine ester-chloroform solution mentioned above, and the mixture thoroughly shaken. Part of the tyrosine ester combines with liberated hydrochloric acid, forming the insoluble hydrochloride. A little of the sodium carbonate solution was added and the shaking continued. The re-

[2] Erlenmeyer, E., *Ann. Chem.*, 1893, cclxxv, 1.

mainder of the cinnamoyl chloride and the carbonate solutions was now alternately added in small amounts; the mixture was constantly shaken and kept cool. The chloroform solution was then dried with sodium sulfate and precipitated with much petroleum ether. The voluminous and cloudy precipitate is at first pasty, but changes gradually into crystalline sediment. This was dissolved again in chloroform, and after repeated precipitation with petroleum ether, finally recrystallized with dilute alcohol. Its melting point is 136°C. (uncorrected). For analysis the substance was then dried at 100°.

0.1700 gm. substance gave 4.7 cc. 0.1 N NH_3.

	Calculated for $C_{20}H_{21}O_4N$:	Found:
N	4.13	3.87

The saponification of the ester was accomplished by simply dissolving the substance in N NaOH. By neutralizing with hydrochloric acid the cinnamoyl tyrosine was precipitated as an amorphous mass. The pure substance, polygonal prisms, obtained by crystallizing from dilute alcohol, melted at 166–167°C. (uncorrected), and was dextrorotatory in alkaline solution. The substance was dried at 100°.

0.2576 gm. substance gave 15.5216 gm., dissolved in about 15 cc. N NaOH: specific gravity 1.0447; percentage 1.6596; length of tube, 1 dm. $\alpha = +0.66$ at 25°C. Therefore $[\alpha]_D = +38.07°$.

0.1973 gm. substance gave 0.5039 gm. CO_2 and 0.0980 gm. H_2O.

0.1256 " " " 3.8 cc. 0.1 N NH_3.

	Calculated for $C_{18}H_{17}O_4N$:	Found:
C	69.42	69.65
H	5.51	5.53
N	4.50	4.24

The cinnamoyl-*l*-tyrosine is readily soluble in alcohol, slightly in ether, and scarcely soluble in water.

As experimental animals, dogs and rabbits were employed. The substances to be tested were used in the form of a weak alkaline carbonate solution and were administered subcutaneously or introduced into the stomach. Only in dogs were the substances given *per os* as powder wrapped in meat. The urine of 24 or 48 hours was concentrated over steam and extracted with alcohol.

TABLE I.

Benzoyl-α-Aminocinnamic Acid.

Animal.	Weight.	Method.	Amount given.	Found in ether extract.	Optically active substances in mother liquor.
	kg.		*gm.*	*gm.*	
Dog	14.6	Subcutaneous.	2.0	0.863	—
"	8.2	"	2.0	1.215	—
"	14.6	*Per os.*	2.0	1.300	—
Rabbit	2.1	Subcutaneous.	0.7	0.321	—
"	2.3	"	1.5	0.722	—
"	2.7	*Per os.*	1.0	0.687	—

TABLE II.

p-Hydroxylbenzoyl-α-Aminocinnamic Acid.

Animal.	Weight.	Method.	Amount given.	Found in ether extract.	Optically active substances in mother liquor.
	kg.		*gm.*	*gm.*	
Dog	8.20	Subcutaneous.	2.0	0.554	—
"	3.10	"	1.0	0.276	—
Rabbit	3.00	"	0.7	—	—
Dog	3.50	*Per os.*	2.0	0.013	—
Rabbit	2.95	" "	1.0	0.120	—
"	3.00	" "	1.25	—	—
"	2.61	" "	1.5	—	—

TABLE III.

Benzoyl-o-Aminocinnamic Acid.

Animal.	Weight.	Method.	Amount given.	Immediately after acidification.	Found in ether extract.	Optically active substances in mother liquor.
	kg.		*gm.*	*gm.*		
Dog	4.5	Subcutaneous.	1.0	0.503	Minimal.	—
"	6.8	"	2.0	1.266	"	—
Rabbit	2.3	"	0.7	0.360	—	—
Dog	4.5	*Per os.*	1.5	0.497	Minimal.	—
Rabbit	2.4	" "	1.0	0.630	—	—

TABLE IV.

Cinnamoyl-l-Tyrosine.

Animal.	Weight.	Method.	Amount given.	Found in ether extract.	Optically active substances in mother liquor.
	kg.		*gm.*	*gm.*	
Dog	12.1	Subcutaneous.	2.0	—	—
"	7.9	*Per os.*	2.0	—	—
"	7.9	" "	2.0	—	—
"	6.7	" "	2.0	—	—
Rabbit	2.7	Subcutaneous.	1.0	0.07	—
"	3.3	"	0.7	—	—
"	2.6	*Per os.*	1.0	0.306	—
"	2.4	Subcutaneous.	0.8	0.245	—
"	2.7	*Per os.*	2.0	0.383	—
"	3.1	" "	2.0	0.572	—
"	3.3	" "	2.0	0.51	—
"	2.7	" "	2.0	—	—

The alcoholic extract was in turn evaporated and the residue dissolved in water. The solution, strongly acidified with hydrochloric acid, was first thoroughly extracted with ether in the Kumagawa-Suto extractor. The resulting acid residue was then shaken with ethyl acetate. From this extract, only urea was isolated. The mother liquors contained no optically active substances. The substances found were identified by their physical properties and especially by determining their melting point. The mixing test with analytically pure substance was performed each time.

The substance found in the ether extract was recrystallized from hot dilute alcohol and purified. The figures given thus indicate the quantity of analytically pure benzoyl-α-aminocinnamic acid.

BIOLOGICAL OBSERVATIONS ON THE FORMATION OF PHENOL.

By MIDORI TSUDJI.

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The formation of phenols in the animal body is a well known fact. They are known as the putrefaction products of the pancreas, muscle, liver, and fibrin.[1] However, the phenols as judged by the reaction with bromine water are not a single substance, but include *p*-cresol, *o*-cresol, *m*-cresol, and phenol. Baumann and Brieger[2] have shown that the phenols produced on putrefaction of the liver are for the most part *p*-cresol, *o*-cresol, and a little phenol. It was obvious that the mother substance of phenol might be tyrosine, and though Baumann failed in one of his experiments, Brieger,[3] who administered tyrosine to a healthy man, was successful in showing by the bromine water test much phenol in the urine. Finally, Weyl,[4] on adding sewer mud to tyrosine, confirmed that its putrefaction product was one of the phenols, indeed *p*-cresol itself. Hence, Baumann,[5] thinking that phenol should arise by oxidation of *p*-cresol, administered *p*-cresol to a dog and *p*-oxybenzoic acid was obtained in the urine; also, after feeding *p*-oxybenzoic acid to the same dog, he obtained phenols in the urine. After subjecting hydroparacumalic acid[6] (*p*-oxyphenolpropionic acid) to putrefactive fermentation with pancreas substance, he obtained *p*-oxyphenylacetic acid, *p*-cresol, and phenol. Since hydroparacumalic acid is the decomposition

[1] Baumann, E., *Z. physiol. Chem.*, 1877, i, 60. Brieger, L., *ibid.*, 1879, iii, 134. Odermatt, W., *J. prakt. Chem.*, 1878, xviii, 249. Nencki, M., Opera omnia, Braunschweig, 1905, i, 404.

[2] Baumann, E., and Brieger, L., *Z. physiol. Chem.*, 1879, iii, 149.

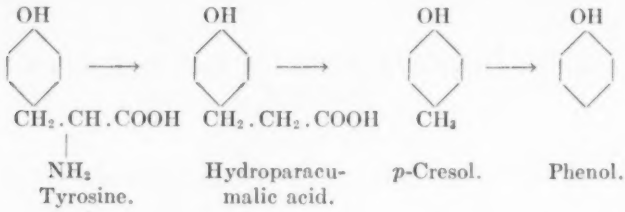
[3] Brieger, L., *Z. physiol. Chem.*, 1878, ii, 241.

[4] Weyl, T., *Z. physiol. Chem.*, 1879, iii, 312.

[5] Baumann, E., *Z. physiol. Chem.*, 1879, iii, 250.

[6] Baumann, E., *Z. physiol. Chem.*, 1880, iv, 304.

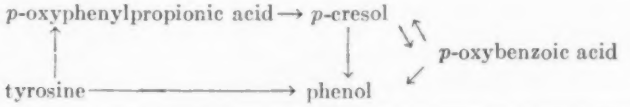
product of tyrosine, Baumann considered the formation of phenol as follows:



Nevertheless, Baumann could not directly derive phenol from *p*-cresol; also, in testing for phenol in the urine of a dog which had been given *p*-oxybenzoic acid, he used the reaction with bromine water. It was therefore not certain whether the decomposition product was phenol or not, as it could also be *p*-cresol. Fortunately an important contribution was furnished by Siegfried and Zimmermann,[7] who, after giving *p*-oxybenzoic acid to dogs, obtained 32 per cent each of phenol and *p*-cresol in the urine.

Although the production of phenol from both *p*-cresol and *p*-oxybenzoic acid has thus been proved, we must not come too readily to the conclusion that phenol is obtained from hydroparacumalic acid, because by fermentation of certain organs containing none of it, phenol is produced.

Judging from the structural formula, the mother substance of phenol seems to be tyrosine, but we must note that phenylalanine is also capable of being the mother substance of phenol. When Baumann put forward his hypothesis, phenylalanine had not yet been identified as a protein constituent. On studying phenol formation by organisms, I found unexpectedly *Bacillus coli communis*, which actively forms phenol. I studied its action on tyrosine and phenylalanine; no phenol was formed from phenylalanine; however, much phenol but no cresol was obtained from tyrosine. From these experimental results, phenol formation may be represented thus:



[7] Siegfried, M., and Zimmermann, R., *Biochem. Z.*, 1912, xlvi, 210.

Baumann assumes hydroparacumalic acid to be concerned in phenol formation, but it must be admitted that there remains room for further investigation. We have among the decomposition products of tyrosine so called tyrosol, *p*-oxyphenyllactic acid, and tyramine; the possibility of these substances being concerned in phenol production must be considered.

EXPERIMENTAL.

Various organisms were examined as to phenol production after being grown for 30 days in bouillon containing 2 per cent peptone (Witte). The methods employed for testing for phenols were: Millon's reaction, bromine water, and ferric salts reaction. The tests were made on distillates derived from the cultivating fluid, which had been acidified by addition of 10 cc. of dilute sulfuric acid and distilled with steam.

TABLE I.

30 Days

Bacillus.	No. of strain.	Phenols.
B. proteus vulgaris	1	−
" " "	2	−
" " "	3	−
" *typhosus*		−
" *paratyphosus*		−
" *coli communis*	1	+++
" " "	2	+
" *subtilis*		−
" *pyocyaneus*		−

The results in Table I show that *Bacillus coli* is the only organism among those tested that produced noteworthy amounts of phenol. In a second investigation, it was found that *Bacillus coli* forms significant amounts of phenol after 2 days, and that its quantity subsequently increases.

To determine whether phenylalanine could yield phenol, the following experiment was made. A nutrient medium containing glycerol and the usual inorganic salts was used with 0.5 gm. of phenylalanine or 1.0 gm. of tyrosine per liter. It was heavily inoculated with *Bacillus coli* from twenty agar slants and then

incubated. After 30 days, the culture was still pure and the mixtures were acidified and distilled. The phenylalanine experiment gave no trace of phenol, while tyrosine gave much. It may be inferred from these results that the phenols come from tyrosine, while *dl*-phenylalanine does not contribute to them. Subsequent experiments made under both aerobic and anaerobic conditions only confirmed the conclusion.

In order to identify the phenol formed from tyrosine, and present in the acid distillates, an attempt was made to convert it into the barium salt of phenylsulfuric acid. The amount obtained was too small for analysis, so that the isolation of phenol itself was next attempted. The distillate was made alkaline with potash, evaporated, acidified with sulfuric acid, and extracted with ether. The ether was evaporated at low temperatures, and on cooling the residue in an ice box, needles of phenol, melting at 42–43°, were readily obtained. The yield was only about 30 mg., but the product showed an unchanged melting point when mixed with pure phenol and gave all the characteristic reactions of the latter substance.

In conclusion, I wish to acknowledge my indebtedness to Professor Sasaki for his interest and advice during the progress of these studies.

A STUDY OF THE PHYSIOLOGY OF ENDOGENOUS URIC ACID.

BY H. F. HÖST.

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(Received for publication, October 28, 1918.)

Our knowledge of the physiology of endogenous uric acid is still incomplete. Aside from questions concerning the accumulation of uric acid in blood, about which opinions differ, little is known of the concentration of uric acid in blood. It is not certain whether the concentration of the endogenous uric acid in blood is a constant factor for the individual, or whether it varies, and if so, whether the variation during the day is regular or irregular.

The amount of endogenous uric acid in the urine has been the subject of many investigations, which have resulted in two different opinions. The majority of the authors emphasize the fact that the output of the endogenous uric acid in 24 hours is constant for the individual (Burian and Schur, Sivén, Hall, Brugsch and Schittenhelm, Brugsch, etc.), while the minority come to the conclusion that the excretion of uric acid on a purine-free diet varies irregularly (Hindhede, Abl).

Some authors are of the opinion that the output of endogenous uric acid is constant for the individual when the diet is the same or nearly the same, but that the excretion is influenced by variations in diet (Umber, etc.).

The present report deals with the determination of uric acid in the urine and blood in seventeen individuals, two of whom were normal, while the remainder were convalescents and patients who had never had symptoms of gout. In addition to the quantity of uric acid the nitrogen output was determined in all the individuals, and in four the hydrogen ion concentration of the urine also was measured.

Method.—The uric acid concentration in blood was determined colorimetrically by Folin's method in the way previously de-

scribed (Höst, 1915, *a*). The method is correct within 10 per cent. The uric acid in urine was determined by the method of Folin and Denis, modified by Benedict. The nitrogen output was determined by the Kjeldahl method. The hydrogen ion concentration of the urine was determined at first by the electrometric method of Hasselbalch, and later by the colorimetric method, which I have already described (Höst, 1915, *b*).

In the present paper I shall report only the experiments with the two normal individuals.

EXPERIMENTAL.

Experiment 1.—Laboratory assistant and gardener, age 31 years, normal. The subject is a well built, healthy man and has never had symptoms of gout. During the investigations at the laboratory of Dr. Hindhede he maintained his weight on 4,200 calories. Throughout the experiments he therefore received this amount of calories, except in the underfeeding and overfeeding periods. The experiments were undertaken to study the influence of great variations in the amount of protein and calories in the diet on the output of endogenous uric acid. The experiment consisted of six periods.

Period 1.—May 8 to 11. Diet: coarse rye bread 1,200 gm. and butter 175 gm. (protein 98 gm., fat 163.5 gm., carbohydrate 600 gm.), equivalent to about 4,250 calories. The subject had lived on this diet since May 4, and thus the uric acid output even from the 1st day of the investigation was in proportion to the diet. On the 4 days of this period the uric acid output varied between 0.84 and 0.88 gm. (Fig. 1), and averaged 0.85 gm.

Period 2.—May 12 to 16. Diet: coarse rye bread 800 gm., butter 295 gm. (protein 67 gm., fat 269.5 gm., carbohydrate 400 gm.), equivalent to about 4,250 calories. In comparison with the first period this period contains about 30 per cent less protein and carbohydrate, while the fat was increased by about 60 gm., so that the caloric value remains the same. On the 1st day the nitrogen and uric acid output diminished considerably. The nitrogen continued to diminish slowly, while the uric acid excretion on the following 5 days had a constant value of 0.75 to 0.76 gm.

Period 3.—May 17 to 18. Diet: coarse rye bread 500 gm., butter 295 gm., dried apples 200 gm., sugar 50 gm. (protein 47.6

gm., fat 269 gm., carbohydrate 433.5 gm.), equivalent to about 4,300 calories. The protein was again reduced by 30 per cent, while the presence of carbohydrate was slightly raised and the fat remained the same. As the curve shows, both the nitrogen and the uric acid output diminished further on this diet. The uric acid excretion on the 1st day was 0.72 gm. and on the 2nd day 0.67 gm. Unfortunately the period was too short to show how much the excretion would have diminished on this diet.

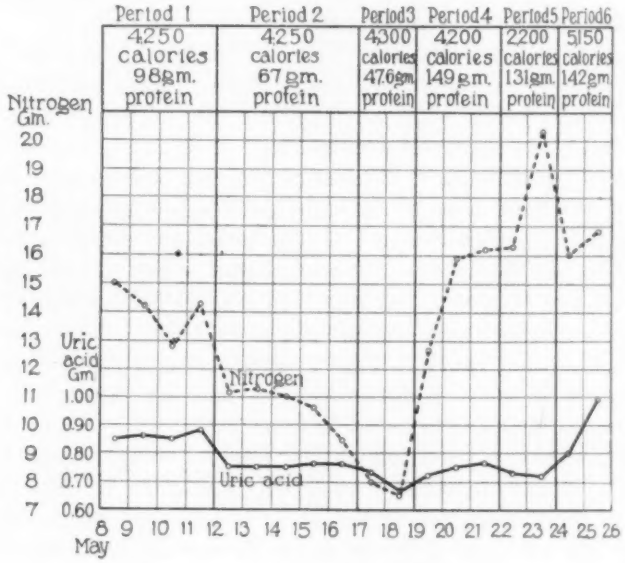

Fig. 1.

Period 4.—May 19 to 21. Diet: milk 1,000 cc., eggs 600 gm., butter 185 gm., and coarse rye bread 185 gm. (protein 149 gm., fat 264 gm., carbohydrate 301 gm.), equivalent to about 4,200 calories. The protein was then increased by about 200 per cent, while the carbohydrate was diminished 20 per cent, and the fat remained the same. On this protein-rich diet the nitrogen excretion immediately increased considerably, while the uric acid output grew slowly, and not before the 2nd and the 3rd days reached the amount of 0.75 and 0.76 gm., respectively (see Fig. 1).

Period 5.—Period 5, May 22 to 23, is one of underfeeding,

which consisted of milk 1,000 cc., eggs 600 gm., coarse rye bread 300 gm. (protein 131 gm., fat 96.5 gm., carbohydrate 185 gm.), equivalent to about 2,200 calories. The protein remained the same, while the fat and carbohydrate were diminished to such a degree, that the caloric value of the diet was lowered one-half. The nitrogen output on the 1st day was unchanged, but the next day it increased to 20.3 gm. Presumably the deficit in the food during the 1st day was made good by the glycogen in the liver, which acted to spare protein, so that the latter did not begin to be metabolized in increasing amount until the 2nd day.

Period 6.—May 24 to 25. Diet: coarse rye bread 1,500 gm., butter 200 gm. (protein 142 gm., fat 187.5 gm., carbohydrate 751 gm.), equivalent to about 5,150 calories. This period represents one of considerable overfeeding. The amount of protein was about the same as in the last period, but the quantity of the fat and especially that of the carbohydrate was much increased. Although the protein was a little increased, the nitrogen output on the 1st day diminished to about 16 gm., which was due to the fact that the organism, having abundance of nitrogen-free foodstuffs, economized with the protein metabolism. The uric acid output increased rapidly (Fig. 1) and the 2nd day reached a value of 0.99 gm. Unfortunately I had no opportunity to continue the investigations on this diet.

The excretion of endogenous uric acid is, in this subject, not constant because it varies considerably according to the composition of the diet as well as the quantity and quality.

During the 5 days of Period 2 the endogenous uric acid excretion was constant within the experimental error of the method. But on the last day of Period 1 the uric acid excretion increased considerably in spite of the fact that the diet was the same as on the former 3 days. The other periods were too short to determine whether the uric acid output was constant on the diet.

The influence of the composition of the diet on the uric acid excretion shows itself in: (*a*) Reduction of the protein and simultaneous increase of the fat and carbohydrate, total caloric value remaining constant, reduce the uric acid output. (*b*) Increase of the protein and simultaneous reduction of the carbohydrates in such a way that the caloric value is the same, increase the uric acid

output. (*c*) Underfeeding due to reduction of the nitrogen-free foodstuffs, the quantity of the protein being the same, effects a reduction of the uric acid excretion. (*d*) Overfeeding due to nitrogen-free foodstuffs, the protein being the same, increases the uric acid output.

From the results of the first four periods, it is seen that the uric acid output is not proportional to the quantity of the protein, the caloric value being the same. While in Period 1 the uric acid excretion is between 0.84 and 0.88 gm. and the protein of the diet is 98 gm., the uric acid output in Period 4 is only 0.75 to 0.76 gm. while the diet contains 149 gm. of protein.

When in Periods 1 and 4 the uric acid output is compared with the protein in the diet, it should be remembered that the protein in these two periods is given in different foodstuffs, in Period 1 essentially in bread, in Period 4 essentially in eggs and milk, and only to a lesser extent in bread.

The reduction of the protein in Period 1 is, as may be seen, due to a reduction of the quantity of the bread; it might therefore be doubtful, whether it is the bread itself or the protein in the bread that influences the uric acid output. That the protein as such is of importance is, however, evident from Period 4, in which the quantity of bread is the same as in Period 3, while the content of protein is much greater. The increase of the uric acid output in the fourth period is, however, not so large as might be expected from the amount of protein, and this fact suggests that the bread may have influenced the uric acid output. The great increase in the uric acid in Period 4 confirms this to some extent. This period represents one of overfeeding, which means increased uric acid output, but the increase the 2nd day is so great that the large quantity of bread, 1,500 gm., no doubt has been of importance in this connection. Unfortunately, I had no opportunity to continue the investigations on the influence of the coarse rye bread on the uric acid excretion.

The uric acid concentration in the blood was determined four times, with the following results:

Date.	Uric acid concentration of blood.		
	I	II	Average.
	mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*
May 10, 3 p.m.....	2.35	2.41	2.38
" 16, 3 "	2.51	2.60	2.60
" 18, 3 "	2.25	2.37	2.31
" 23, 3 "	2.20	2.34	2.28

In spite of the fact that the uric acid excretion during 24 hours, as a result of changes in the composition of the food, varies greatly, the uric acid in the blood is constant within the experimental error of the method.

Experiment 2.—Physician, age 32 years, normal. The subject of the experiment has never had any serious illness or symptoms of gout. During the entire period of investigations laboratory work was carried on. The uric acid output was tested every 24 hours and in shorter periods. The experiment is divided into three periods.

Preliminary Period.—April 5 to 9. As is evident from Fig. 2 the uric acid output in the preliminary period is between 0.44 and 0.54 gm. each day. The nitrogen excretion shows in this period a complete accordance with the uric acid output.

Period 1.—In Period 1, April 10 to 30, in which the food was entirely purine-free, the uric acid output and the nitrogen output in the first 6 days were determined every 6 hours; namely, from 8 a.m. to 2 p.m., from 2 p.m. to 8 p.m., from 8 p.m. to 2 a.m., and from 2 a.m. to 8 a.m. The urine of the first 2 nights was collected from 8 p.m. to 8 a.m., but the results were with a view to correct proportions in the curve marked off as if divided in the periods 8 to 2 and 2 to 8.

It is evident from Fig. 3 that the uric acid output during the first 4 days is greatest between 8 a.m. and 2 p.m., less in the afternoon, and least at night. There seems to be no marked difference in the uric acid output before and after midnight. On the 14th some purine-containing food and tea and coffee were taken. The influence of this is seen immediately in the uric acid output, which is considerably increased in the night of the 14th to the 15th and in the forenoon of the 15th. The influ-

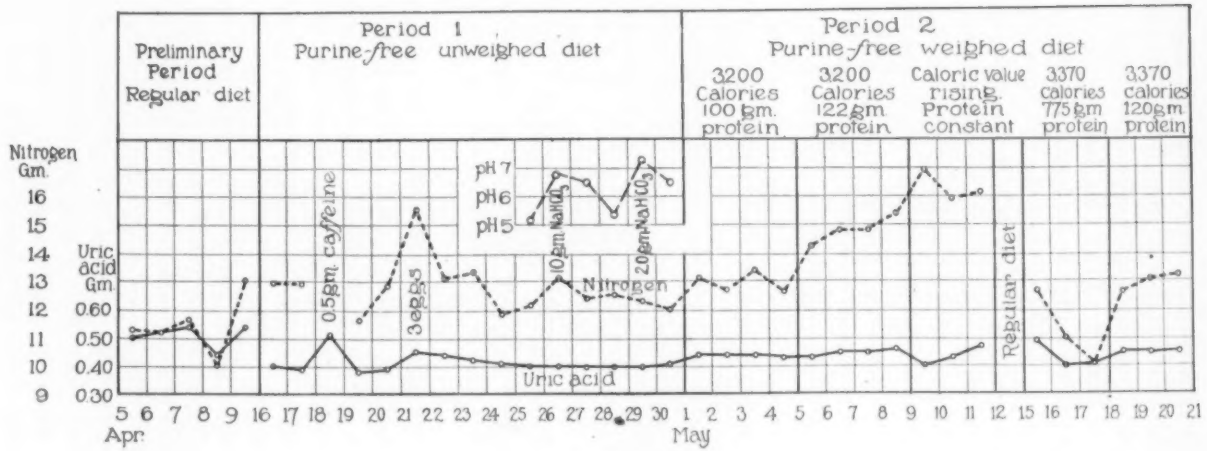
Preliminary Period Regular diet
Period 1 Purine-free unweighed diet
Period 2 Purine-free weighed diet
3200 Calories 100 gm. protein
3200 Calories 122 gm protein
Caloric value rising. Protein constant
3370 calories 775 gm protein
3370 calories 120 gm protein
Nitrogen Gm.
Uric acid Gm.
0.5 gm. caffeine
3 eggs
pH 7
pH 6
pH 5
10 gm NaHCO3
20 gm NaHCO3
Nitrogen
Uric acid
Regular diet
5 6 7 8 9 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 1 2 3 4 5 6 7 8 9 10 11 12 15 16 17 18 19 20 21
Apr.
May

FIG. 2.

ence of the absorbed purines on the uric acid excretion was apparent until the afternoon of the 15th, that is 15 to 20 hours later. During the remainder of this period the urine was analyzed every 24 hours (Fig. 2). From the 16th to the 18th, the uric acid output was about 0.40 gm.; on the day 0.5 gm. of caffeine was given it increased to 0.51 gm., and the following day it decreased to about 0.40 gm. again. On the 21st the diet contained three eggs, in consequence of which the uric acid output immediately increased to 0.45 gm.; after this the excretion slowly diminished until on the 5th day it reached 0.40 gm. We observed the different way in which the uric acid output increased when caffeine and eggs were added to the food. During this period the influence of alkalies was also studied; on the 26th, and 29th, 10 and 20

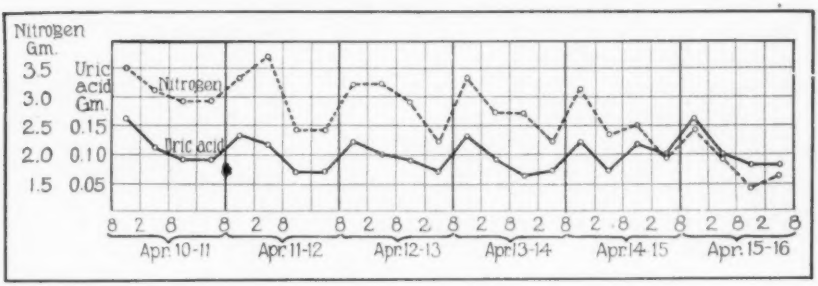

FIG. 3.

gm. of sodium bicarbonate, respectively, were given. The pH of urine increased, as Fig. 2 shows, to 6.80 and 7.30, respectively, but the uric acid excretion was not influenced in the least. Except for the increase of the excretion on account of caffeine and eggs, this period is characterized by a constant endogenous uric acid output at 0.40 gm.

Period 2.—In Period 2, from May 1 to 23, the diet consisted of weighed quantities of purine-free foodstuffs. In this period the influence of the composition of the diet and of the digestion on the uric acid excretion was tested more exactly. In the first 4 days the diet consisted of milk 1,000 cc., graham bread 450 gm., butter 100 gm., and two eggs (protein 100 gm., fat 167 gm., carbohydate 321 gm.), equivalent to 3,200 calories. Upon beginning this diet the uric acid output increased to 0.44 gm. and remained

almost unchanged for all the 4 days. The cause of this increase cannot be determined with certainty since the composition and the quantity of the diet in the former were not constant. The subject of the experiment was of the opinion that the special quantity of bread and also that of the total amount of calories were increased in this period, which was probably the cause of the increased uric acid excretion. In the next 4 days of the period, May 5 to 8, the diet consisted of milk 1,000 cc., graham bread 375 gm., cheese 100 gm., butter 88 gm., and six eggs (protein 122 gm., fat 170.5 gm., and carbohydrate 284 gm.), equivalent to 3,200 calories. The quantity of calories was therefore the same, while the presence of protein increased from 100 to 122 gm., and that of the carbohydrate decreased correspondingly.

TABLE I.

Date.	Diet.	Protein.	Fat.	Carbohydrate.	Calories.
1915		*gm.*	*gm.*	*gm.*	
May 9	Bread 225 gm., butter 58 gm., cheese 135 gm., 6 eggs, milk 1,000 cc.....	111	145	260	2,670
" 10	Bread 410 gm., butter 115 gm., cheese 100 gm., 4 eggs, milk 1,000 cc.....	111	183	301	3,310
" 11	Bread 450 gm., butter 130 gm., cheese 135 gm., 2 eggs, milk 1,500 cc.....	121	208	362	3,835

The amount of fat was practically the same. Coincident with this change in the diet the uric acid output increased to about 0.45 gm. and during these 4 days averaged 9 mg. more than in the first 4 days. In the following 3 days, May 9 to 11, the amount of calories was varied by means of fat and carbohydrate, while the protein was about the same. The diet is given in Table I.

On the 1st day, May 9, the amount of calories was 2,670, an underfeeding which is at once accompanied by a decrease to 0.40 gm. in the uric acid output. The following 2 days the amount of calories increased to 3,310 and 3,835, respectively, and at the same time the uric acid excretion increased to 0.43 and 0.47 gm., respectively (Fig. 2). The increase of calories is due to a steady increase of fat and carbohydrate (Table I). The last day also the amount of protein rose slightly, from 111 to 121 gm., but this

was too slight to explain the considerable increase of uric acid output from the 2nd to the 3rd day. From May 12 to 14 the experiments were interrupted; the diet on these days was regular and not purine-free. During the following 6 days, May 15 to 20, the relation between the uric acid output and the protein of the diet was studied by means of greater variation in the amount of protein than in Experiment 1. The diet is given in Table II.

While in Experiment 1 a certain amount of carbohydrate was replaced with an isocaloric amount of protein, in Experiment 2 fat is replaced by corresponding quantities of protein, the amount of carbohydrate and calories being the same. On the first 3 days (May 15 to 17, Fig. 2) the diet contained 77.5 gm. of protein. The 1st day the uric acid output was rather great, doubtless owing to residual exogenous uric acid. The following 2 days the

TABLE II.

Date.	Diet.	Protein.	Fat.	Carbohydrate.	Calories.
1915		*gm.*	*gm.*	*gm.*	
May 15 to 17	Graham bread 450 gm., butter 200 gm., milk 1,000 cc.....	77.5	220	276	3,372
" 18 " 20	Graham bread 450 gm., butter 136 gm., milk 1,000 cc., 6 eggs	120	198	276	3,370

uric acid excretion was 0.40 and 0.41 gm., respectively. From May 18 to 20 the amount of protein was increased to 120 gm. (*i.e.* about 50 per cent), and at the same time the uric acid output increased to 0.45 gm. and was exactly the same on the 2 following days.

During the last 3 days of the experiment, May 21 to 23, the diet was the same as on the preceding days, but from 7 a.m. to 11 p.m. the uric acid and the nitrogen output were determined every 2 hours in order to study the effect of the meals. The food was divided into three equal parts, taken at 7 a.m., 1 p.m., and 7 p.m. The uric acid output during the 3 days is parallel during each of the days (Fig. 4); the output is greatest in the morning and in the forenoon; it is slightly less during the remainder of the day and is least in the evening and at night. The daily output is given in detail in Fig. 4.

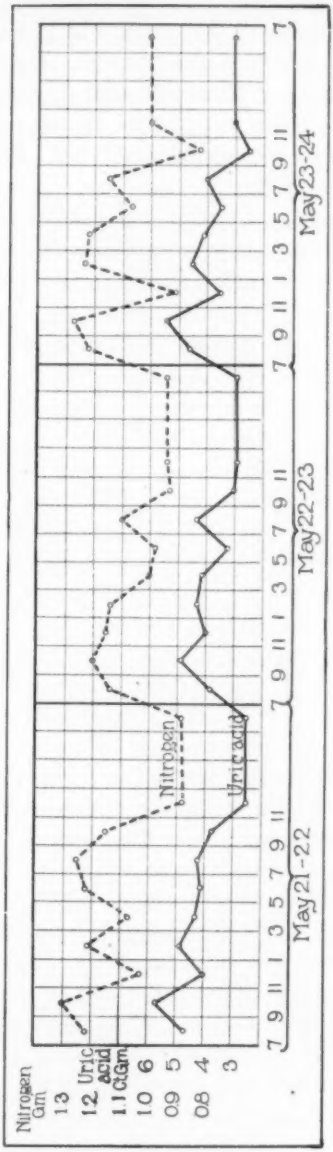
Nitrogen Gm
13
12
11
10
09
08
Uric acid Gm
6
5
4
3
Nitrogen
Uric acid
7 9 11 1 3 5 7 9 11
May 21-22
7 9 11 1 3 5 7 9 11
May 22-23
7 9 11 1 3 5 7 9 11
May 23-24
7

Fig. 4.

The uric acid output was relatively great even between 7 and 9 a.m., and reached its maximum from 9 to 11 a.m., that is 2 to 4 hours after the first meal. From 11 a.m. to 1 p.m. the output decreased considerably, but increased again between 1 and 3 p.m. after the meal at 1. From 3 to 7 p.m. the excretion diminished, increased from 7 to 9 p.m. after the meal at 7, and decreased again at night.

Apart from the increases caused by the meals at 1 and 7 p.m. the curve is characterized by the fact that it rose sharply in the morning, reached its maximum early in the forenoon and then fell slowly and somewhat evenly down to a minimum at night. That the meal at 7 a.m. was not distinctly marked on the uric

TABLE III.

Date.	Uric acid concentration of blood.		
	I	II	Average.
1915	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
Apr. 22, 5.30 p.m.	1.94		1.94
" 26, 10.30 a.m.	2.10	1.94	2.02
" 26, 5.30 "	1.94	1.94	1.94
May 3, 5.30 "	2.04	2.04	2.04
" 11, 5.30 "	1.92		1.92
" 16, 5.30 "	2.03	2.10	2.07
" 20, 12.00 m.	2.00	2.12	2.06

acid curve, is undoubtedly due to the fact that the uric acid output had already begun to rise at this time of the day.

The uric acid concentration was determined seven times. The results are given in Table III from which it is evident that the endogenous uric acid concentration in the blood in this experiment was constant within the limits of error of the method, in spite of the fact that the food had been changed considerably both in quantity and quality, and the uric acid output had in consequence varied from 0.40 to 0.47 gm. in 24 hours.

The uric acid concentration of the blood was determined twice on the same day, before and 7 hours after the administration of 10 and 20 gm. of sodium bicarbonate, respectively. As is apparent from Fig. 2, the alkali taken produces just as little change in the amount of the uric acid in the blood as in the urine.

CONCLUSIONS.

From the results on the two normal individuals reported here and fifteen other subjects who were convalescents and patients the following conclusions are drawn.

In none of the seventeen subjects is the uric acid output for 24 hours constant. But with a fixed diet and under similar conditions the uric acid output in a few subjects was constant, while in the majority of those examined it was extremely irregular and showed variations from day to day up to 80 per cent.

But even in the individuals whose output of uric acid was most regular, the output was dependent on several factors, of which variations in the diet are the most important. In every increase or decrease of the caloric value of the food beyond a certain minimum, the uric acid output was always changed in the same direction. This took place whether the caloric value was varied by means of protein, fat, or carbohydrate; the change in the uric acid output is, however, greater when the amount of calories is varied by means of protein than by nitrogen-free food elements. With a constant food caloric value the uric acid output depended to a certain extent on the food protein, so that a change in the latter beyond a certain minimum always produced a corresponding change in the uric acid output.

The excretion of nitrogen and the hydrogen ion concentration of the urine were without influence on the endogenous uric acid output. On the other hand, increase of body temperature produced a considerable increase in the uric acid output.

The endogenous uric acid output in 24 hours varied between 0.27 and 0.99 gm.; the last value is obtained in a normal person on an extensive bread diet.

The amount of the endogenous uric acid in the blood varied to a great extent, the minimum being less than 0.50, the maximum being 2.68 gm. per 100 cc. of blood, but is in the case of each individual constant within the limit of error of the method (10 per cent). In 70 per cent of the subjects the uric acid concentration is between 0.01 and 0.02 gm. per 100 cc. of blood.

The investigations of previous workers show that the uric acid is not metabolized in the organism, and the experiments prove that the concentration of the endogenous uric acid in the blood

in each individual is constant, the endogenous uric acid output becomes a direct expression for the uric acid formation, and we find the characteristic fact, that while in some individuals there is a regular endogenous uric acid production in others it is extremely irregular.

Definite conclusions regarding synthetic uric acid formation cannot be drawn from the experiments. The uric acid output and accordingly the uric acid production also depend to a certain extent on the quantity and quality of the purine-free diet. But the changes in the uric acid excretion, which are produced by variations in the food are, however, not so great that they may not be explained as depending on an increased or diminished operation on the part of the digestive organs. In this respect also the results of the uric acid determinations for the 2 hour periods agree, as they make it probable that a not inconsiderable part of the endogenous uric acid comes from metabolism in the tissue of the digestive glands.

It is characteristic of the endogenous uric acid metabolism that the concentration of uric acid in the blood is constant in the individual, but that it varies considerably in different individuals, and that the stability of the uric acid production varies greatly in different individuals. While some individuals produce and excrete daily a constant or almost constant amount of uric acid, the uric acid production in others is extremely irregular, so that the uric acid output in 24 hours is subject to relatively great variations.

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STUDIES ON THE BEHAVIOR OF INULIN IN THE ANIMAL BODY.

PRELIMINARY PAPER.

APPLICATION OF THE BENEDICT METHOD TO THE ESTIMATION OF LEVULOSE AND INULIN.

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(Received for publication, February 20, 1919.)

The experiments reported in the following paper were carried out by the author as a series of tests on the application of the Benedict (1) modification of the Lewis-Benedict method for sugar determination to the estimation of levulose and inulin, preliminary to a study of the behavior of the latter carbohydrate. Because these tests have made apparent the influence of certain factors which might easily be neglected in applications of this method to the analysis of *sugar solutions*, it has been considered worth while to publish the results.

Preparation of Standards.

The sample of glucose, analyzed by the gravimetric method of Munson and Walker, was 96.8 per cent pure; the levulose, by the method of Allihn, 98.2 per cent; while the samples of inulin used gave, after hydrolysis, reduction values equivalent to 94.7 and 90.5 per cent of that for the calculated amount of levulose; the moisture content of the samples was, however, 5.2 and 9.3 per cent.

Since pure picramic acid was not available, a sample of the acid assaying approximately 30 per cent of the estimated color was purified by recrystallization from boiling water according to the method of Egerer (2). With this product, the picramic acid standard, prepared according to Benedict's directions for the estimation of sugar in urine (1), was compared in a Duboscq colorim-

eter with the standard prepared by treating 1 mg. of the (96.8 per cent pure) glucose in 4 cc. of water with 4 cc. of the picrate-picric acid solution and 1 cc. of 20 per cent sodium carbonate, heating in a boiling water bath for 10 minutes, cooling, and diluting to 25 cc. When this glucose standard was set at 15 mm., the picramic acid matched it at 14.6 mm., or the picramic acid standard was almost exactly equal in color to a solution representing 1 mg. of 100 per cent pure glucose, treated as described above and diluted to 25 cc.

Estimation of Levulose.

When this picramic acid standard was set at 15 mm., 1 mg. samples of levulose, treated as in the preparation of the glucose standard described above, were found to match it at 14.9 to

TABLE I.

Estimation of Levulose in Presence of Inulin.
(Technique as for Glucose Standard.)

Weight of levulose added.	Weight of inulin added.	Time heated.*	Average reading.	Increase due to inulin.
mg.	*mg.*	*min.*	*mm.*	*mm.*
1.0	0.0	15	15.0	—
1.0	0.9	15	15.1	—
1.0	1.8	15	15.0	—
1.0	2.7	15	14.8	0.2
1.0	4.5	15	13.9	1.1

* After adding Na_2CO_3.

15 mm. Since the levulose sample used was approximately 98.2 per cent pure, levulose must give a color very nearly equivalent to that from 102 per cent of its weight of glucose.

It was found, on experiment, that if quantities of inulin equivalent to three times the weight of sugar usually used for such a determination were heated with the alkaline picrate solution for 15 minutes, no deepening of the color produced by heating the picrate with the carbonate alone resulted; and also, as shown by Table I, that the addition of small quantities of inulin to the levulose samples caused no appreciable change in the color produced by the levulose in the alkaline picrate solutions. Hence, it seemed practicable to use this method for the estimation of levulose and of levulose in the presence of small amounts of inulin.

Effect of Heating the Sugars with Picric Acid.

The quantitative estimation of inulin by hydrolysis with the picrate-picric acid solution and determination of the levulose formed by heating it with sodium carbonate in the same solution was next attempted. This method was suggested by Rose's (3) determination of sucrose. Samples of inulin, 0.9 mg. each, were heated with the picrate-picric acid solution for periods varying from 10 minutes to 1 hour. To avoid the effect of the increased

TABLE II.

*Hydrolysis of Inulin by Picric Acid.**
(Readings Represent Duplicate Determinations.)

Date.	Weight of inulin.	Time heated, acid solution.†	Range of readings.	Average reading.	Inulin (Benedict, colorimetric.)	Inulin (Allihn, gravimetric.)
1918	*mg.*	*min.*	*mm.*	*mm.*	*per cent*	*per cent*
Nov. 11	1.0	10	17.3–17.5	17.4	77.5	90.5
" 11	1.0	15	16.2–16.4	16.3	82.8	90.5
" 11	1.0	20	15.5–15.8	15.7	85.9	90.5
" 7	0.9	25	16.1–16.2	16.15	92.8	90.5
" 7	0.9	30	15.7–15.8	15.75	95.1	90.5
" 7	0.9	35	15.1–15.3	15.2	98.5	90.5
" 13	0.9	20	15.1–15.2	15.15	98.6	94.5
" 13	9	30	13.1–13.2	13.15	114.4	94.5
1919						
Jan. 7*	0.9	40	16.4–16.9	16.65	90.9	94.5
" 7*	0.9	50	16.2–16.5	16.35	92.0	94.5
" 7*	0.9	60	16.0–16.2	16.1	93.7	94.5

* Determinations made with a sample of picric acid which had been purified by the method of Folin and Doisy; for the others commercial samples were used.

† Samples were, in every case, heated for 10 minutes after adding the sodium carbonate.

concentration of the picrate which resulted, the solutions were then cooled, made up to the original volume, the carbonate was added, and the tubes were again heated. As the results shown in Table II indicate, there was a deepening of the color developed on 10 minutes heating with the carbonate which increased with the time of heating in the acid picrate solution.

There seemed to be two plausible explanations for these results; namely, that the increased color was due either to the prolonged

action of the hot concentrated picric acid on the monosaccharide formed in the hydrolysis, or to the action of possible impurities in the picric acid used. A quantity of picric acid was, therefore, purified according to the method of Folin and Doisy (4), and a series of tests on the effect of heating glucose and levulose with the picrate-picric acid was made, each test being accompanied

TABLE III.

Effect of Heating Glucose and Levulose with the Picrate-Picric Acid Solution.
(Readings Represent Duplicate Determinations.)

Date.	Sugar.	Weight of sample.	Time heated, acid solution.*	Picric acid.	Average reading.	Control reading.†
1918		*mg.*	*min.*		*mm.*	*mm.*
Dec. 9	Glucose.	1.0	15	Purified.	14.7	15.2
" 11	"	1.0	20	"	14.4	15.2
Nov. 29	"	1.0	25	"	13.8	15.1
Dec. 9	"	1.0	30	"	13.3	15.2
Nov. 29	"	1.0	35	"	13.4	15.1
Dec. 9	"	1.0	45	"	13.3	15.2
Nov. 29	"	1.0	60	"	13.2	15.1
Dec. 9	"	1.0	60	"	13.1	15.2
" 13	"	1.0	60	"	13.2	15.2
" 11	"	1.0	60	"	13.2	15.2
" 12	"	1.0	60	Commercial.	13.2	15.2
" 13	"	1.0	70	"	12.5	15.2
Nov. 26	Levulose.	1.0	10	Purified.	14.3	15.1
" 26	"	1.0	20	"	13.4	15.1
" 26	"	1.0	40	"	13.3	15.1
Dec. 13	"	1.0	50	"	13.0	15.0
" 11	"	1.0	60	"	13.0	15.0
" 13	"	1.0	50	Commercial.	13.2	15.0

* Samples heated, in every case, for 10 minutes after adding the sodium carbonate.

† Control readings represent the readings of the same solutions of glucose and levulose which were heated only after adding the carbonate.

by a determination of the value of the sugar solution used according to the technique described for the preparation of the glucose standard (for detailed results see Table III). Since it was evident from these experiments that heating glucose and levulose with the picrate-picric acid solutions resulted in an increase in the depth of the final color varying with the time of heating in the

acid solution, the idea of estimating the value of inulin solutions by hydrolysis with the picrate-picric acid and colorimetric determination of the levulose in the same solution was abandoned.

Hydrolysis with Hydrochloric Acid.

Effect of Chlorides.

The next series of experiments dealt with the estimation of inulin by hydrolysis with hydrochloric acid, followed by a determination of the levulose by the Benedict method. To 90 mg. samples of inulin in 20 cc. of water was added HCl to give concentrations equivalent, respectively, to 0.5 and 1 per cent actual HCl. The resulting solutions were covered with watch-glasses to minimize evaporation, and heated over boiling water baths for varying lengths of time, heating for 1 hour with 0.5 per cent HCl being found to give the most nearly uniform results. The solutions were then cooled, neutralized exactly to methyl red with sodium hydroxide, made up to 100 cc., and 1 cc. portions analyzed according to the procedure recommended by Benedict for the preparation of the glucose standard for use in the determination of sugar in urine.

The results were so low, judging them from the analyses by the Allihn method, that their accuracy was questioned. The next step was, therefore, to study the effect of addition of HCl to solutions of glucose and levulose, using the same concentrations and technique as in the inulin determinations, and heating the solutions the usual 10 minutes for the development of the picramic acid color. It was evident that this procedure also gave results which were too low (Table IV).

In order to determine whether or not this effect was due to the destruction of the carbohydrate by the HCl, or to the interference of the NaCl formed by the neutralization of the acid with the development of the color, 20 cc. of the 1 per cent HCl were exactly neutralized with NaOH, made up to 100 cc., and amounts of this NaCl solution equivalent to those which had been present in the previous determinations were added to 1 mg. samples of glucose, and these analyzed in the usual way. The results were again low, indicating that the NaCl was in some way responsible. In order to eliminate the effect of possible impurities in the acid or alkali, a

TABLE IV.

Effect of NaCl, KCl, *and* HCl *on Sugar Values as Determined by the Benedict Method.*

Date.	Sugar.	Sample.	Reagent added (1 cc. to 1 mg. sugar).	Time heated, acid solution.	Time heated, alkaline solution.	Picric acid.	No. of determinations.	Range of readings.	Average reading.	Control reading.*
		mg.		*min.*	*min.*			*mm.*	*mm.*	*mm.*
Nov. 27, 1918	Glucose.	1.0	HCl†	60	10	Purified.	4	16.2–16.5	16.4	15.3
" 27, "	"	1.0	HCl†	60	10	Commercial.	2	15.9–16.3	16.2	15.1
" 29, "	"	1.0	NaCl‡	—	10	Purified.	2	16.6–16.8	16.7	15.2
Dec. 9, "	"	1.0	KCl§	—	10	"	2	16.4–16.5	16.4	15.2
" 12, " Jan. 13, 1919	"	1.0	NaCl, 0.65%‖	—	10	"	9	16.8–17.3	17.1	15.2
" 15, " " 3, "										
" 15, " " 21, "	"	1.0	NaCl, 0.65%‖	—	15	"	6	15.0–15.2	15.1	15.1
Nov. 24, 1918	Levulose.	1.0	HCl†	60	10	Commercial.	2	17.1–17.3	17.1	15.0
" 27, "	"	1.0	HCl†	60	10	Purified.	2	16.4–16.7	16.6	15.0
Dec. 11, "	"	1.0	NaCl‡	—	10	"	4	17.0–17.4	17.1	15.2
" 13, " Jan. 15, 1919	"	1.0	NaCl, 0.65%‖	—	10	"	6	16.9–17.2	17.0	15.1
" 15, " " 27, "	"	1.0	NaCl, 0.65%‖	—	15	"	4	14.9–15.4	15.2	15.2

* Control readings are those made on the same sugar solutions to which no salts had been added.

† 100 mg. of the sugar heated for 1 hour with 20 cc. of 1 per cent HCl, cooled, neutralized with NaOH, and made up to 100 cc.; 1 cc. portions taken for analysis.

‡ NaCl made by neutralizing 20 cc. of 1 per cent HCl with NaOH and making up to 100 cc.

§ KCl " " " 20 " " 1 " " HCl " KOH " " " " 100 "

‖ Kahlbaum's *zur Analyse* NaCl.

concentration of Kahlbaum's *zur Analyse* NaCl corresponding to that formed by the neutralization of the acid, namely, 3.3 mg. per cc., was substituted. Again the results were the same.

These experiments were repeated between twenty-five and thirty times in order to find, if possible, some error in the technique. An interruption led to the increase in the time of heating the alkaline picrate solution on one day from the 10 minutes prescribed by Benedict to 15 minutes. It was found that in this case the colors of the solutions containing the NaCl exactly matched those of the control determinations, making it evident that, in some way, the effect of the NaCl was to retard the rate of development of the color; a difficulty which, as is indicated by Table IV, could easily be overcome by continuing the heating of the alkaline solution for 15 minutes. Determinations of inulin by HCl hydrolysis, followed by 15 minutes heating in the alkaline picrate solution, gave results which were comparative, but which were slightly lower than the total carbohydrate content of the samples as determined by the Allihn method.

Effect of Other Salts.

At this point an investigation of the effect of the presence of some of the other salts of the blood was undertaken. Solutions of 0.1 M disodium hydrogen phosphate, 0.1 M sodium dihydrogen phosphate, a buffer mixture of the two in concentrations corresponding to a hydrogen ion concentration of pH = 7, and a solution of NaCl representing a normal blood concentration of this salt (0.65 per cent NaCl) were made up. Determination of the value of 1 mg. samples of glucose to which 1 cc. portions of these solutions had been added were then made, using the technique described above for the preparation of the glucose standard. It was found that the dihydrogen phosphate tended to retard the rate of development of the color, while the disodium phosphate showed no effect at the end of 10 minutes heating, but, after 15 minutes, tended to give colors deeper than those of the glucose controls which contained no salts. That these effects might be due to changes in the hydrogen ion concentration seemed to be indicated by the fact that the buffer solution had no effect either on the rate of development of the color or the depth of the color

TABLE V.

Effect of Various Salts Normally Present in Blood on Determinations of Glucose by the Benedict Method.*

Date.	Sample.	Technique.	Added salt.	Time heated. †	No. of determinations.	Range of readings.	Control reading.
1919	*mg.*			*min.*		*mm.*	*mm.*
Jan. 20, " 22	1.0	As for glucose standard.	NaH_2PO_4 (1 cc. 0.1M)	10	4	16.9–17.1	15.2
" 21	1.0	"	NaH_2PO_4 (1 cc 0.1M)	15	2	15.0–15.1	15.1
" 20, " 22	1.0	"	Na_2HPO_4 (1 cc. 0.1M)	10	4	14.9–15.1	15.2
" 21	1.0	"	Na_2HPO_4 (1 cc. 0.1M)	15	4	14.4–14.9	15.0
" 20, " 22	1.0	"	Buffer (1 cc. 0.1M)	10	4	15.2–15.4	15.2
" 21	1.0	"	Buffer (1 cc. 0.1M)	15	2	15.1–15.1	15.1
" 20, " 22	1.0	"	Buffer + 0.65% NaCl (0.5 cc. each)	10	4	17.5–17.9	15.1
" 27	4 mg. in 2 cc.	As for blood.	NaH_2PO_4 (2 cc. 0.1M)	10	2	15.0–15.1	15.2
" 27	4 " " 2 "	"	Na_2HPO_4 (2 cc. 0.1M)	10	2	14.9–15.0	15.2
" 27	4 " " 2 "	"	NaCl (2 cc. 0.65%)	10	2	15.0–15.0	15.2
" 27	4 " " 2 "	"	NaCl (1 cc. 0.65%) + buffer (1cc 0.1 M)	10	2	14.9–15.0	15.2

* Results of similar determinations of levulose are almost exactly identical with those for glucose.

† Time of heating after adding the sodium carbonate solution.

developed. Addition of 0.5 cc. of the buffer solution and 0.5 cc. of the NaCl solution to 1 mg. samples of glucose and levulose gave, repeatedly, a much greater retardation at the end of 10 minutes heating than 1 cc. of the NaCl alone, although 15 minutes heating was still sufficient for complete development of the color.

Effect of Adding Salts Directly to Blood.

The analyses reported above were made according to the procedure recommended by Benedict for the preparation of the glucose standard to be used in the determination of sugar in the urine. In order to ascertain the extent to which the factor of salt concentration might affect determinations of blood sugar, analyses of rabbit's blood, to which these salts had been added in concentrations of 1 cc. of the salt solutions described above to each cc. of blood, were made according to his procedure for blood. No effect of the salts was observed; the readings obtained after 10 and after 15 minutes heating with the salt solutions were exactly equal to those obtained with the blood alone, using the same technique. Addition of glucose and levulose to the blood samples resulted in their quantitative recovery, except that where the concentrations were considerably higher than in normal blood there seemed, after 15 minutes heating, to be a recovery of amounts slightly greater than those added. This may possibly be explained by some of the findings of Addis and Shevky (5), concerning the effect of prolonged heating on varying concentrations of sugar.

It was found also that values for the pure sugars as determined by the technique for blood, both after 10 and after 15 minutes heating of the sugar in the alkaline picrate solutions, were unchanged by the addition of these salts. It seems, therefore, that the larger concentration[1] of the picrate-picric acid solution called for by the method for the determination of blood sugar overcomes completely the effect of these salts.

[1] The method as applied to the determination of blood sugar involves the heating of 0.64 cc. of the sample equivalent approximately to 0.64 mg. of sugar, with $\frac{5}{25} \times 19$ cc. = 6.08 cc. of the picrate-picric acid solution minus the fraction used up in the precipitation of the proteins of the blood, as against the 4 cc. used for 1 mg. of glucose in the preparation of the standard for urine.

Time of heating after adding the sodium carbonate solution.

SUMMARY.

Benedict's modification of the Lewis-Benedict method has been used successfully for the determination of levulose, and of levulose in the presence of inulin.

When inulin is hydrolyzed by the picrate-picric acid solution, products are formed, which, on heating after the addition of the carbonate, give colors which increase in depth with the time of heating in the acid solution, and tend to become more intense than those from equivalent quantities of levulose. The color developed by glucose and levulose solutions which have been heated with the acid picrate before the addition of the carbonate is also more intense than that given by the same amounts of the sugar solutions treated by the usual procedure.

When the quantities of the picrate-picric acid solution recommended by Benedict for the preparation of the glucose standard for the determination of sugar in urine are used, the addition of small amounts of sodium chloride and sodium dihydrogen phosphate to samples of glucose and levulose cause very appreciable retardation in the rate of color development. Therefore, when it is desired to estimate the monosaccharide formed by acid hydrolysis of a more complex carbohydrate, the influence of the salt formed by the neutralization of the acid must be taken into consideration. Interference of these salts may be avoided by increasing the time of heating with the carbonate to 15 minutes or by using the higher concentration of the picrate-picric acid called for in the determination of blood sugar.

The writer is indebted to Professor H. B. Lewis of this laboratory for helpful suggestions and criticisms.

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DIGESTIBILITY OF BACON.

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Rubner,[1] 40 years ago, published figures for the digestibility of bacon fat which showed low utilization, a loss in the feces of 17.4 per cent of the fat eaten. This is so different from figures published then and since,[2,3] for the utilization of soft fats including lard, and so different also from the every day experience of the satisfactory character of bacon as food, that it seemed desirable to perform other digestibility experiments with bacon. Rubner's own explanation of the extensive loss was that the bacon was eaten in large lumps and the fat was imbedded in connective tissue. It may be also that in his experiment bacon was eaten raw, though he does not say so. He found large pieces of fat in the feces, not disintegrated.

Our experiment was carried out in the spring of 1916 by three young women[4] students in two periods of 3 days each, with an interval of 3 days in which ordinary food was eaten. Three other students later took part in a shorter supplementary experiment.

The diet consisted of bacon, shredded wheat biscuit, orange juice, and a small amount of sugar. The bacon, obtained through the courtesy of Swift and Company, was specially selected in order to be as nearly uniform as possible and to have a large percentage of fat. In Period 1 it was cooked only slightly and in

[1] Rubner, M., *Z. Biol.*, 1879, xv, 170. Quoted by von Noorden, C., *Metabolism and practical medicine*, 1907, i, 55.

[2] Cf. Langworthy, C. F., and Holmes, A. D., *U. S. Dept. Agric., Bull. 310*, 1915.

[3] Cf. Smith, C. A., Miller, R. J., and Hawk, P. B., *J. Biol. Chem.*, 1915, xxiii, 505.

[4] The careful cooperation of all these students is gratefully acknowledged, especially Miss Mary Rising who did a part of the analytical work.

Period 2 much more thoroughly. The average raw weight eaten per day was 350 gm. during Period 1 and 790 gm. during Period 2. Since the slightly cooked bacon lost on the average 66 per cent of its weight in cooking and the much cooked 80 per cent, the cooked bacon eaten averaged 119 gm. and 158 gm. for the two periods, giving 83 and 102 gm. of fat. In preparing the thoroughly cooked material the process was continued till the steam had almost ceased bubbling off, but no charring had commenced.

To obtain the composition of the cooked bacon a number of analyses for fat and nitrogen were made on similar samples. Fat was determined as usual by ether extraction, and nitrogen by Kjeldahl on the fat-free residue. Close duplicates could not be obtained on the somewhat variable material, especially for the

TABLE I.

Composition of Cooked Bacon.

	Loss of wt. in cooking.	Fat.	Nitrogen.
	per cent	*per cent*	*per cent*
Slightly cooked.	56–58	74.5	2.5
	65–69	71.5	3.2
	79	64.5	4.5
Much cooked.	82	61.9	4.8
	87	60.3	4.7

slightly cooked, but since twelve different determinations were made on it the resulting figures should be reliable. The averages are given in Table I and on two curves plotted in Fig. 1. To find the amount of bacon fat consumed at each meal the loss of weight in the cooking was noted and the corresponding percentages of fat were read from the curve.

The small amount of fat in shredded wheat biscuits was computed from published analyses,[5] since they seemed sufficiently uniform (1.4 per cent). As the nitrogen from this source was a somewhat large proportion of the nitrogen of the diet, it was determined as usual and found to be 1.73 per cent. The orange juice nitrogen

[5] *U. S. Dept. Agric., Off. Exp. Stat. Bull. 28*, 1906; *Farmers' Bull. 249*, 1906; *Modern Hospital*, 1915, iv, 165.

was taken as 0.09 per cent.[6] The amount and composition of food eaten are given in Table II.

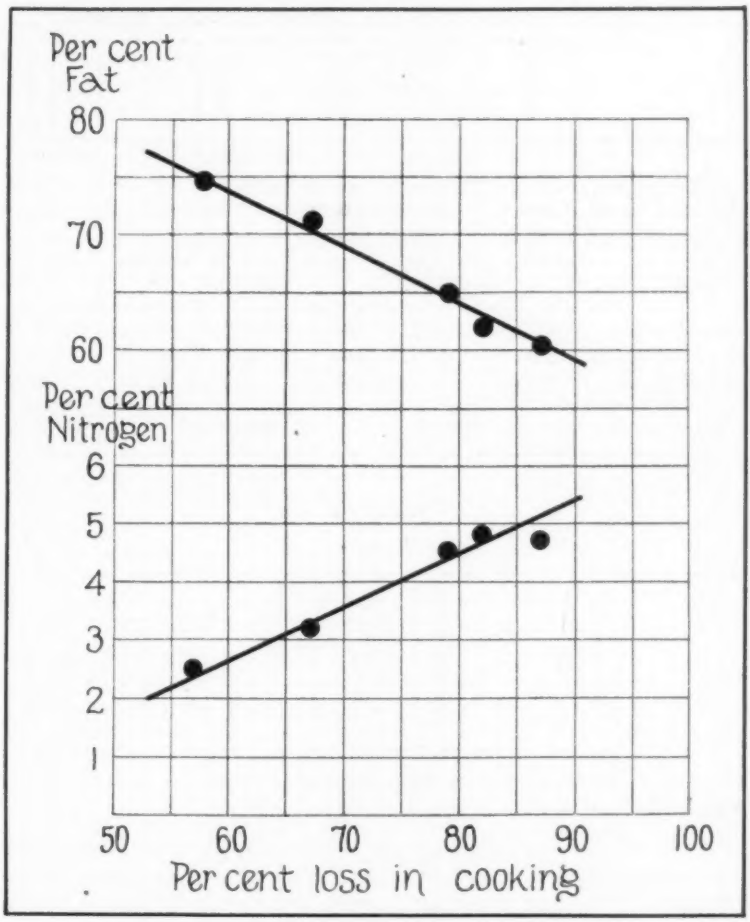

FIG. 1. Curves of composition of cooked bacon.

To identify the feces of the test period charcoal was taken at the first and last meal. Daily collection of the feces was made

[6] Thorpe, E., Dictionary of applied chemistry, London and New York, 2nd edition, 1913, iv, 21.

TABLE II.

Food.

	Subject M.			Subject B.			Subject R.		
	Quantity.	Fat.	Nitrogen.	Quantity.	Fat.	Nitrogen.	Quantity.	Fat.	Nitrogen.
		gm.	gm.		gm.	gm.		gm.	gm.
Cooked bacon...	355 gm.	261	11.9	348 gm.	235	11.0			
Shredded wheat.	390 "	5	6.8	490 "	7	8.6			
Orange juice.....	1,080 cc.		0.9	1,080 cc.		0.9			
Total.....	.....	266	19.6		242	20.5			
Cooked bacon...	493 gm.	316	21.8	498 gm.	322	21.4	435 gm.	283	19.2
Shredded wheat.	390 "	5	6.8	364 "	5	6.3	468 "	6	8.1
Orange juice.....	1,265 cc.		1.1	1,450 cc.		1.3	1,407 cc.		1.2
Total.....	.....	321	29.7		327	29.0		289	28.5

TABLE III.

Feces.

	Subject M.					Subject B.					Subject R.				
	Wt. of stool.	Total fat.	Fatty acid.	Neutral fat.	Nitrogen.	Wt. of stool.	Total fat.	Fatty acid.	Neutral fat.	Nitrogen.	Wt. of stool.	Total fat.	Fatty acid.	Neutral fat.	Nitrogen.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
	94	7.1	3.0	4.1	1.39	49	3.1	1.4	1.7	0.60					
Period 1.	60	3.6	1.1	2.5	0.59	149	8.6	3.7	4.9	1.80					
	108	5.0	2.5	2.5	1.55	67	4.4	2.1	2.3	0.77					
	27	1.4	0.6	0.8	0.48	61	3.1	1.2	1.9	0.85					
Total.....	...	17.1	7.2	9.9	4.01		19.2	8.4	10.8	4.02					
	47	3.0	1.0	2.0	0.88	30	1.8	0.6	1.2	0.80	170	8.6	5.1	3.5	2.80
Period 2.	114	4.4	1.7	2.7	1.86	158	6.1	2.2	3.9	3.22	92	2.9	0.9	2.0	1.19
	95	5.5	1.9	3.6	1.91	131	5.3	2.2	3.1	1.94	131	5.6	2.5	3.1	2.01
						39	2.5	1.0	1.5	1.04					
Total.....	...	12.9	4.6	8.3	4.65		15.7	6.0	9.7	7.00		17.1	8.5	8.6	6.00

from the time of the first to the last appearance of the dark coloration. Daily feces were weighed, thoroughly mixed, and sampled for analysis. The analyses were made on a wet basis. The Saxon method[7] was used for the determination of fat. According to this, the moist sample is treated with water, hydrochloric acid, alcohol, and ether, the top ethereal layer containing the fat and the fatty acid repeatedly blown off, the ether evaporated, and the fat weighed. The fat was then titrated with 0.05 N sodium hydroxide to determine the free fatty acid of the feces, as was done by Smith, Miller, and Hawk.[3] The fatty acid represents the fat which has been acted upon by the enzyme but not absorbed; *i.e.*, digested but not utilized. The total fat represents the fatty acid and the

TABLE IV.

Summary. Coefficients of Utilization.

	Subject M.		Subject B.		Subject R.	
	Period.		Period.		Period.	
	1	2	1	2	1	2
Fat eaten, *gm.*	266	321	242	327		290
Total fat in feces, *gm.*	17.1	12.9	19.2	15.7		17.1
Neutral fat, *gm.*	9.9	8.3	10.8	9.7		8.5
Fat digested (split), *per cent*	**96.1**	**98.4**	**96.6**	**96.0**		**96.8**
" utilized, *per cent*	**93.6**	**95.9**	**92.1**	**95.1**		**94.1**
Nitrogen eaten, *gm.*	19.3	29.7	20.6	29.0		29.4
" in feces, *gm.*	4.01	4.65	4.02	7.00		6.00
" utilized, *per cent*	**79.2**	**84.4**	**80.4**	**75.8**		**79.7**

unsplit or neutral fat. Results for fat and nitrogen are given in Table III. The percentage of total fat occurring as free fatty acid is about the same in this experiment as in that of Smith, Miller, and Hawk,[3] theirs being 41.6 per cent and 42.4 per cent in our experiment.

The average percentage of digestion of the fat of slightly cooked bacon was 96.3, and of the much cooked 97.0 (Table IV); while the average percentage of the utilization of the slightly cooked bacon was 92.8, and of the much cooked 95.0, approximately the same as the figures for most fats given by Hawk, by Langworthy

[7] Saxon, G. J., *J. Biol. Chem.*, 1914, xvii, 99.

and Holmes, and other investigators. There is little difference between the two ways of cooking. Especially noteworthy, however, is the fact that our utilization of bacon fat is much higher than Rubner's, who found the fat utilization to be only 82.6 per cent. As mentioned above, he speaks of finding unchanged pieces of bacon in the feces. In our experiment very minute particles were found, which were apparently unchanged shredded wheat but not fat.

These results were confirmed later by a single 3 day experiment in which three students ate the same diet, using moderately cooked bacon and determining the fat only in the mixed feces of the period. The coefficients of utilization were 96.2, 95.7, and 96.5 per cent.

The nitrogen of the diet was not so well utilized—79.8 per cent on the average in Period 1 and 80.0 per cent in Period 2. These low figures, however, are undoubtedly due not to the bacon nitrogen but to the shredded wheat. In the diet of Subject M, for example, in Period 1, 34.7 per cent of the nitrogen came from the shredded wheat, the coefficient of digestibility of which has been reported[8] as only 57.7 per cent. Only 65.3 per cent of the nitrogen came from the meat and the small amount of orange juice. A computation, $79.8 - (34.7 \times 0.577) = X \times 65.3$, shows that X, or the digestibility of the bacon and orange juice nitrogen, is 92 per cent. This is not far from the usual figure for meat protein.

SUMMARY.

The coefficient of digestibility of much and slightly cooked bacon fat is found to average 96.7 per cent, approximately the same as that for other soft fat and much higher than the 82.6 per cent reported by Rubner for bacon eaten in large pieces (raw?). The nitrogen also is as well digested as that of other meat.

[8] Maine Agricultural Experiment Station quoted by Street. J. P., *Modern Hospital*. 1915. iv. 165.

COLORIMETRIC DETERMINATION OF THE HYDROGEN ION CONCENTRATION IN SMALL QUANTITIES OF SOLUTION.*

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The hydrogen ion concentration (1) of many solutions and of culture media is readily determined by means of buffer solutions and suitable indicators. The usual procedure, in determining colorimetrically the hydrogen ion concentration, requires a considerable volume of solution. Frequently the quantity of solution available in experiments upon plants or animals is extremely limited in amount. It is of interest, therefore, to ascertain which modifications of the colorimetric method are best suited for the determination of the hydrogen ion concentration in small volumes of solution.

Recently, several such modifications have been used by different investigators. In determining the pH values of small quantities of plant sap, Wagner (2) has made use of lacmosol (3) as an indicator in a microchemical method. The indicator was found to give excellent results between pH 4.2 to 6.0. A very small quantity of the sap was placed on a slide to which was added the same quantity of lacmosol as had been added to a series of slides each bearing buffer solutions of known pH value. The concentration of the indicator was the same for each slide. The matching of the colors of the indicator was at first carried out by means of the spectroscope. The use of the spectroscope was found so laborious that the colors were compared preferably by the use of two microscopes with twenty magnifications, whereby differences as small as 0.03 in pH could be detected.

The spectroscope has been used by Tingle (4) to determine the neutral point (in titrations for total acid) in solutions in which the natural color of the solution masks the color of the indicator. In such cases, much larger quantities of indicator are required than would be desirable in ordinary

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titrations. The conclusion is reached, save in the case of methyl orange, that it is inadvisable to use the spectroscope for titrations except when the liquid is so colored that no other means can be used for detecting the end-point.

In determining the hydrogen ion concentration of the sap of succulents, Miss Hemple (5) has found it impossible in many cases to employ the colorimetric method as drawn up by Sörensen (6) owing to the color present in the sap. Accordingly, two forms of the electrometric method (7, 8) were employed by her as an accurate means of determining the pH value. However great the exactness of these methods, it was frequently found advisable to employ a method whereby it was possible in the course of the investigation to ascertain rapidly and without waste of material the condition of the sap of a plant at any interval of time. At the suggestion of Professor Sörensen she examined various indicator papers as produced at the Carlsberg laboratory and found that lacmoid paper with a range of pH 3.8 to 6.0 was suitable for the purposes of the experiment at hand. A drop of the unknown solution was applied to a piece of lacmoid paper and the color produced upon the paper was then matched with the colors produced by drops of buffer solutions of known hydrogen ion concentration.

In comparing the electrometric with the lacmoid paper values for the pH of the sap of succulents, Hemple found that rarely did a divergence of 0.4 to 0.5 in pH occur between both methods and that the difference was generally only 0.2 to 0.1.

The use of lacmoid paper, while it does not give an exact means of measuring the hydrogen ion concentration, does afford a rapid and convenient method of obtaining an approximate value. I have found that frequently in bacteriological, as well as in plant physiological work, it is desirable to know quickly and without waste or contamination of a solution, the approximate hydrogen ion concentration. Accordingly the various indicators that are now most widely used in colorimetric determinations were investigated with the view of obtaining indicator papers that would cover the usual working range of pH.

It was soon found that many of the sulfonephthalein (1) group of indicators, that give such excellent results when used in solutions, do not lend themselves readily for the preparation of stable indicator papers unless extreme precautions are taken to keep them in an atmosphere free from carbon dioxide and to protect them as much as possible from the light. Indicator papers, prepared with bromocresol purple, bromothymol blue, phenol red, phenolphthalein, or with mixtures (1) of these, may, under ordinary conditions, even by their evanescence or rather reversibility of

color change, serve to give us a clue as to the approximate range in which the hydrogen ion concentration value lies.

Lacmoid paper was prepared according to the method described by Glaser (9). Varying results are obtained according to the care taken in the preparation of the paper. A small quantity of Mercks lacmoid was digested with 96 per cent alcohol on a water bath and the solution was then filtered. The alcoholic filtrate was used directly in the preparation of the indicator papers. If it is desired, the filtrate may be evaporated to dryness in a vacuum at a low temperature. It was thought best to prepare both the red and the blue papers in order to read the more acid end of the range on the blue paper and the less acid end of the range on the red paper. Various intermediate colored papers were also prepared.

To prepare the blue lacmoid paper, dilute sulfuric acid was added to the alcoholic lacmoid solution until the strips of white filter paper were no longer colored blue but became red. If too much acid has not been added, the paper becomes blue on drying. The sulfuric acid should be added carefully and test papers made upon each small addition of acid in order to add just the correct amount of the acid.

To prepare the red lacmoid paper, dilute sulfuric acid was added to the alcoholic solution of the purified lacmoid until the test papers made from the solution appeared rose-red after several hours of drying. If too much acid has been added, then the sensitivity of the paper is decreased.

The papers were suspended upon strings. They usually require to be drawn a second time through the indicator solution, once they have become dry, in order to give the paper an even tone of color. The drying should not be hastened and should be carried on preferably in a carbon dioxide-free atmosphere over soda lime. The depth of color selected depends upon the ability of the investigator to distinguish color differences, and, as a rule, the lighter shades appear to be preferable to the darker shades. The success in preparing sensitive indicator paper depends also to a large degree on the quality of filter paper selected. Strips of a good grade of quantitative filter paper about 1 cm. in width are very convenient. The ordinary filter paper, that has a mottled appearance due to depressions in its surface, cannot be depended

upon to give uniform results. It is advisable to prepare trial indicator papers from several lacmoid solutions that give the red or the blue indicator papers in order to be able to make a selection for sensitivity.

The method of using indicator paper seems to be of sufficient importance to warrant explanation. The procedure followed by us has been to lay, with forceps, the indicator paper upon a piece of filter paper and then to allow a drop of the solution, the pH of which is to be determined, to fall upon the indicator paper. In a similar manner, once the range of pH has been approximately located, a set of standards is prepared by allowing a drop of buffer solutions of known pH to fall upon the indicator paper strips. The strips are immediately placed in a carbon dioxide-free atmosphere over soda lime to dry slowly. The comparison of the color caused by the solution of unknown pH with the colors caused by the buffer solutions is made best when the papers have dried, though it is advisable to observe the papers at intervals in order to be aware of undue changes in color, should any occur. The reading or comparison of colors is made best away from the central part of the drop and not too close to the edge of color change, as here the diffusion processes may give incorrect results. By coating the dried papers with a thin layer of a good grade of paraffin, free from appreciable amounts of acid, more permanent standards may be secured.

With a view toward completing the more acid end of the pH scale, methyl orange paper was prepared with a range of pH 2.4 to 3.8. Strips of a good grade of quantitative filter paper were drawn through a filtered aqueous solution of the indicator and dried on strings. The lighter yellow shades of the indicator paper were preferable to the darker shades as the color changes were the more easily distinguishable.

Bromophenol blue indicator paper with a range of pH 3.4 to 4.6 was prepared by drawing the strips of filter paper through the indicator solution (1) until a uniform blue color was obtained.

Alizarin sodium sulfonate indicator paper with a range of pH 4.0 to 6.0 was prepared by dissolving the indicator in pure distilled water containing a small quantity of alcohol and then proceeding with the strips of filter paper as previously described. The sensitivity of the indicator paper may be somewhat increased by a small addition of dilute sulfuric acid.

An aqueous solution of neutral red pH 7 to 9 may be used to prepare indicator paper for this range, though other papers for this range are much needed.

Azolitmin indicator paper serves to cover the range of pH 6.2 to 8.0. Much of the criticism recently directed against the use of indicator papers has been in connection with the use of litmus in determining the degree of soil acidity. The sensitivity of various blue litmus papers of commerce for the determination of soil acidity has been tested by various investigators (10, 11), with the result that litmus paper determinations of soil acidity are considered unreliable unless unusual precautions are taken.

The writer has found that when the soil is extracted with as little water as possible (12) in order not to dilute the buffer substances more than necessary, and the soil extract is centrifuged free from most of the colloidal material, that the drop method upon azolitmin paper gives a fairly close approximation to the pH value as obtained by the colorimetric method of Sörensen.

Unfortunately much of the litmus paper of commerce is prepared from litmus products of varying degrees of purity, and the product may differ according to the method followed in obtaining it. The litmus of commerce contains, on an average, about 4 to 5 per cent of azolitmin.

A Merck's preparation of azolitmin was dissolved in pure distilled water containing a very small quantity of dilute sodium hydroxide and then the color was adjusted to the shades of the indicator paper desired by successive small additions of dilute oxalic acid.

The concentration of the indicator solutions used in the preparation of the sulfonephthalein papers was approximately of the strength described by Clark and Lubs (1). The other indicator solutions were prepared by diluting concentrated solutions of the indicators until strips of the filter paper gave the desired tint.

For this purpose, strips of indicator paper were prepared from the concentrated solutions and tested. In this manner the dilutions were continued until the dried indicator papers from the indicator solution showed the highest sensitivity when tested with buffer solutions of known pH value.

The perfection of a suitable series of indicator paper standards awaits only the development of the possibilities of the ever growing series (13) of useful indicators (14) now at our disposal.

We have found it convenient, as well as advisable, when dealing with very small volumes of solutions that have been tested by the indicator paper method, to have a check upon the obtained pH values in another way. Such a method consists in placing one or more drops of the solution, whose pH value is being tested, in a cavity of a porcelain spot plate and by placing in other cavities of the plate the same quantity of buffer solutions of known pH value, and then adding the same concentration of indicator in each cavity. The indicator may be diluted on the spot plate and is conveniently added to the small quantities of solution by means of a platinum loop which can be used as a stirring agent, or the diluted indicator can be added from a dropping bottle and the platinum loop used to mix the indicator with the solution. The indicators of Clark and Lubs (1) are most excellent for this method. The color of the solution that is being tested is then compared with the colors developed in the buffer solutions of known pH value. It is understood, however, that whenever enough solution is available and the greatest degree of accuracy is desired, the usual Sörensen colorimetric method and preferably the electrometric method should be used.

SUMMARY.

The indicator paper method, when used with precaution, because of its rapidity and the small volumes of solution required in testing for the pH value, deserves greater use than it has received in the past. The supplementary use of the spot plate method adds to the reliability of the indicator paper determinations.

Although the electrometric or the usual Sörensen colorimetric methods are recognized as being superior, there are cases in which such refined methods either cannot be applied or require too much time. With the described methods it is usually possible to obtain a pH value differing 0.4 to 0.2 pH or even less from those obtained by the more exact methods.

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A RAPID METHOD FOR THE ESTIMATION OF UREA IN URINE.

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(Received for publication, March 26, 1919.)

The method, which is an adaptation of a procedure used in this laboratory for testing the strength of urease preparations, consists in treating a small quantity of urine in a centrifuge tube with an approximately neutral phosphate solution and with urease. After the urea has been decomposed a protein precipitant is added, the tube centrifuged, and an aliquot of the clear supernatant liquid is Nesslerized and compared with a standard.

The amount of urine chosen was 1 cc., and the phosphate solution made of such strength that 1 cc. of it would neutralize the ammonia formed from 1 cc. of concentrated urine.

We have used all manner of urease preparations and have come to the conclusion that finely ground jack bean powder is the most satisfactory, since it is easily prepared and keeps well. Moreover, the powder prepared in this laboratory contains no ammonia and forms none unless subjected to the action of alkali, as in the Marshall-Van Slyke-Cullen aeration method.[1]

The powder has the disadvantage of being difficult to measure out, but if moistened with alcohol it can be moulded readily into tablets which possess enough coherence for handling, but which disintegrate rapidly when added to an analysis.

To hasten the action of the enzyme the test is warmed momentarily to about 50°C., either by shaking over a small flame or by placing in a water bath, and is then allowed to cool for 20 minutes so that it will reach room temperature before the aliquot is taken.

[1] Marshall, E. K., Jr., *J. Biol. Chem.*, 1913, xv, 493. Marshall, E. K., Jr., and Davis, D. M., *ibid.*, 1914, xviii, 53. Van Slyke, D. D., and Cullen, G. E., *ibid.*, 1914, xix, 211; 1916, xxiv, 117.

During the warming no appreciable amount of evaporation will take place unless the material is shaken to the upper part of the centrifuge tube, but some slight evaporation will occur during the centrifuging. To avoid this chance for error, and to make it easier to mix the contents after adding the protein precipitant, tightly fitting cork stoppers which have been soaked in beeswax are used. Care must be taken to cut off the tops of these stoppers, if necessary, so that they will not strike the centrifuge head while in motion.

The precipitant used is acidified potassiomercuric iodide. This forms a precipitate which settles very quickly on centrifuging, and this reagent is the most logical to use where the solution is to be Nesslerized afterwards.

Acidified potassiomercuric iodide solution liberates iodine on standing, but this decomposition can be prevented by the addition of a small amount of sodium bisulfite. Unless the solution is kept tightly stoppered the bisulfite will have to be added from time to time. Whenever free iodine is present it will be plainly indicated by its action on the starch in the urease tablets.

After adding the potassiomercuric iodide the solution must be mixed, which is done by placing the thumb on the stopper and inverting. After mixing, the stopper should be removed for an instant to allow the carbon dioxide liberated by the acid to escape. Centrifuging will usually take about 1 minute. After centrifuging, if it is desired, the analysis can be left for several days before completion, if tightly stoppered. The amount of supernatant liquid most likely to yield 1 mg. of ammonia nitrogen is 1 cc., but if the Nesslerized solution is too dark or too light there will be enough liquid left in the centrifuge tube for another Nesslerization.

Centrifuge tubes that have been used in an analysis are washed with a small test-tube brush covered with soap, and are then rinsed with a solution of potassium iodide and sodium hydroxide to remove the last traces of mercury. *This is absolutely essential*, otherwise the enzyme will be poisoned when the tube is next used for an analysis. The stoppers need only to be rinsed in water and dried with a towel after use, as the enzyme does not come in contact with them during its action. Pipettes that have been in contact with solutions containing mercury must be rinsed with the alkaline potassium iodide before being used to pipette urine or phosphate solution.

Preparation of Reagents.

Phosphate Solution.—Dissolve 111 gm. of $Na_2HPO_4.2H_2O$ and 85 gm. of KH_2PO_4 in about 400 cc. of warm water and dilute to a volume of 500 cc. The solution can be conveniently kept in a bottle fitted with a stopper through which passes a 1 cc. Ostwald pipette, dipping into the liquid not more than 1 or 2 cm. This pipette, as well as others used in the analysis, should be calibrated.

Urease Tablets.—Grind about 50 gm. of jack beans, first in a coffee mill and afterward in a large porcelain mortar, until nearly as fine as wheat flour. Sift gently through cheese-cloth to remove the coarser particles. Moisten the sifted material with a small amount of 95 per cent alcohol and immediately press into a hard rubber tablet mould for making 1 grain tablets. After smoothing and pressing out, the tablets are allowed to dry for several days and are kept in a stoppered test-tube. Two of these tablets, the amount used for an analysis, will weigh about 0.1 gm. and form approximately 11 mg. of ammonia nitrogen in 5 minutes at 20°C. when treated with an excess of phosphate-urea solution. The most practical way, however, of testing their enzyme strength is by the regular method for the analysis of urea in urine described in this paper, using a concentrated sample of urine, and allowing the enzyme to act for 10, 15, and 20 minute intervals after the preliminary heating. The results are then compared. Urease tablets prepared in this laboratory have been found to require 15 minutes plus the preliminary heating to hydrolyze the urea in 1 cc. of urine completely, but to be on the safe side the time that the enzyme is allowed to act has been lengthened to 20 minutes.

Acidified Potassiomercuric Iodide Solution.—To 100 cc. of the potassiomercuric iodide solution of Folin and Denis[2] add 50 cc. of concentrated hydrochloric acid, dilute to 1 liter volume, add 1 gm. of sodium bisulfite, and keep in a stoppered bottle fitted with an accurate 3 cc. pipette.

[2] Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 479.

Procedure.

Into a clean, dry 15 cc. centrifuge tube, which is not sharply constricted, carefully pipette 1 cc. of urine[3] and 1 cc. of the phosphate solution, using Ostwald pipettes. Add two urease tablets, stopper, and shake gently over a small Bunsen flame until the contents of the tube feel just uncomfortably hot to the hand (or warm by allowing the tube to stand for 3 or 4 minutes in water heated to 55°C.). The material should become well mixed during this heating. Allow the tube to stand in a rack for 20 minutes. Now add exactly 3 cc. of the acid potassiomercuric iodide solution, stopper tightly, invert, and shake violently to mix. Remove the stopper for an instant to relieve the gas pressure and centrifuge for 1 or 2 minutes. According to the concentration of the urine used, pipette 0.5, 1, or 2 cc. of the supernatant liquid into a 100 cc. volumetric flask, dilute, Nesslerize with 15 cc. of the Nessler solution of Folin and Denis,[2] make up to volume, and compare in a colorimeter with a 1 mg. standard Nesslerized at the same time. The reading of the standard divided by the reading of the unknown and multiplied by 10, 5, or 2.5, according to whether 0.5, 1, or 2 cc. have been taken as the aliquot, will give the urea and ammonia nitrogen in the original urine in mg. per cc. or gm. per liter. The values for the urea alone can be calculated by subtracting the corresponding figures for ammonia.[4]

Blanks.—To be certain that the method is being properly carried out it may be well for the analyst to run an experiment using, instead of 1 cc. of urine, 2 cc. of an ammonium sulfate solution containing exactly 2 mg. of nitrogen. After adding the reagents and centrifuging, 3 cc. of the supernatant liquid are Nesslerized to 100 cc. volume and compared in the colorimeter with the usual 1 mg. standard. The reading of the blank should agree with the reading of the standard to within about 1 per cent.

To make certain that the reagents contain no ammonia a blank is run, using distilled water instead of urine. Approximately neu-

[3] If the urine is extremely concentrated it will be advisable to use only 0.5 cc., bearing in mind that the total volume of the analysis in this case will be 4.5 cc. instead of 5 cc.

[4] Folin, O., and Bell, R. D., *J. Biol. Chem.*, 1917, xxix, 329. Sumner, J. B., *ibid.*, 1918, xxxiv, 37.

tralize and Nesslerize to 10 cc. volume 1 cc. of the supernatant liquid. If the reagents are free from ammonia the blank will show no color other than that due to the Nessler solution.

In Table I are figures obtained by running parallel determinations by the new method and by the Marshall-Van Slyke-Cullen aeration method.[5]

TABLE I.

Urea and Ammonia Nitrogen in Gm. per Liter of Urine.

		New method.	Marshall-Van Slyke-Cullen method.
1	Normal	2.04	2.01
2	"	2.39	2.42
3	"	6.03	6.08
4	"	4.51	4.50
5	"	6.95	6.95
6	"	5.02	5.03
7	"	4.20	4.16
8	Diabetic	5.88	5.68
9	"	4.48	4.48
10	"	5.24	5.35
11	Nephritic	8.73	8.55
12	"	6.17	6.25
13	"	6.21	6.13
14	"	15.15	15.04
15	"	5.89	5.90
16	"	11.56	11.52
17	"	4.90	4.95

CONCLUSION.

A method has been devised for the estimation of urea in urine which is rapid, accurate, and simple.

We wish to express our thanks to Mr. Hubbard and Mr. Blau of the Clifton Springs Sanitarium for furnishing us with pathological urines.

[5] The figures obtained by the aeration method have been corrected for the ammonia nitrogen evolved by the action of potassium carbonate on the urease tablets during aeration. Under the conditions of the experiment the amount was 0.04 mg.

LYSINE AS A HYDROLYTIC PRODUCT OF HORDEIN.*

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(Received for publication, February 28, 1919.)

Hordein is the alcohol-soluble protein of barley, *Hordeum vulgare*. Determinations of the hydrolytic products of this protein have been made by Osborne and Clapp[1] and by Kleinschmitt.[2] These investigators isolated the basic amino-acids by the direct method of Kossel and found no evidence that hordein contains lysine. The complexity of Kossel's method makes it extremely difficult to isolate lysine when this amino-acid is present in but small quantities in proteins. This difficulty is shown by the results obtained in the earlier analyses of gliadin, the alcohol-soluble protein of wheat, which failed to reveal the presence of lysine. Later work by Osborne and Leavenworth[3] resulted in the isolation of about 0.15 per cent of lysine from gliadin. Further work by Osborne, Van Slyke, Leavenworth, and Vinograd[4] resulted in the isolation of 0.64 per cent of lysine while the indirect method of Van Slyke indicated the presence of 1.21 per cent of lysine in gliadin. A determination of the free amino nitrogen of gliadin by the method of Van Slyke and Birchard[5] verified the latter result. That gliadin contains lysine was also indicated by the observation[6] that white rats grow at a slow rate for a long time when gliadin is the only source of protein in the diet, while proteins which do not contain lysine fail to promote growth.

Nutrition experiments described by Osborne and Mendel[6] show that hordein also promotes growth at a slow rate, which indicates

[1] Osborne, T. B., and Clapp, S. H., *Am. J. Physiol.*, 1907, xix, 117.

[2] Kleinschmitt, A., *Z. physiol. Chem.*, 1907–08, liv, 110.

[3] Osborne, T. B., and Leavenworth, C. S., *J. Biol. Chem.*, 1913, xiv, 481.

[4] Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, 1915, xxii, 259.

[5] Van Slyke, D. D., and Birchard, F. J., *J. Biol. Chem.*, 1913–14, xvi, 539.

[6] Osborne, T. B., and Mendel, L. B., *Z. physiol. Chem.*, 1912, lxxx, 307.

that it contains lysine. The results of the present investigation corroborate this observation.

The hordein used in this investigation was prepared from barley flour by the method of Osborne.[7] The protein was prepare by Mr. Gersdorff of this Laboratory. It was analyzed by the Van Slyke method and duplicate determinations indicated the presence of 0.77 and 1.01 per cent of lysine respectively. A free amino nitrogen determination made on hordein by the method of Van Slyke and Birchard[5] also indicated the presence of 1.01 per cent of lysine.

The sulfur determination on the bases precipitated by phosphotungstic acid showed the presence of at least 1.18 per cent of cystine in hordein. This figure is undoubtedly too low since the cystine is partly decomposed during the hydrolysis of the protein. These results, as well as those previously reported, are given below. It is interesting to note how closely these figures agree with the latest results obtained on gliadin, which are also given.

Basic Amino-Acids in Hordein and Gliadin.

	Amino-acid from 100 gm. of protein.			
	Hordein.			Gliadin,[4] Van Slyke method.*
	Osborne and Clapp,[1] Kossel method.	Kleinschmitt,[2] Kossel method.	Present analysis, Van Slyke method.	
	gm.	gm.	gm.	gm.
Cystine	Not isolated.	Not isolated.	1.18	1.17
Arginine	2.16	3.14	2.89	2.97
Histidine	1.28	0.51	2.14	2.19
Lysine	0.00	0.00	1.01	1.21

* Percentage of cystine estimated from cystine nitrogen obtained by Van Slyke method.

EXPERIMENTAL.

Analysis of Hordein by the Van Slyke Method.—Duplicate 3 gm. samples were used, each equivalent to 2.8311 gm. of moisture- and ash-free protein, and containing 16.92 per cent of nitrogen. Each sample, therefore, contained 0.4790 gm. of nitrogen. The

[7] Osborne, T. B., *J. Am. Chem. Soc.*, 1895, xvii, 539.

protein was dissolved in 100 cc. of 20 per cent hydrochloric acid and hydrolyzed by boiling the solution for 24 hours. The phosphotungstates of the bases were decomposed by the amyl alcohol-ether method. The results are given in Tables I and II.

TABLE I.

Analysis of Hordein, Van Slyke Method. Nitrogen Corrected for Solubility of Bases.

	I	II	I	II	Average.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Amide N	0.1156	0.1129	24.13	23.57	23.85
Humin N adsorbed by lime	0.0041	0.0040	0.85	0.84	0.84
" N in amyl alcohol extract	0.0011	0.0015	0.23	0.32	0.28
Cystine N	0.0039	0.0039	0.81	0.81	0.81
Arginine N	0.0250	0.0264	5.22	5.51	5.37
Histidine N	0.0184	0.0164	3.84	3.42	3.63
Lysine N	0.0042	0.0055	0.88	1.15	1.01
Amino N of filtrate	0.2355	0.2373	49.16	49.54	49.35
Non-amino N of filtrate	0.0703	0.0657	14.68	13.71	14.20
Total N regained	0.4781	0.4736	99.80	98.87	99.34

TABLE II.

Basic Amino-Acids in Hordein. Calculated from Van Slyke Analysis.

Amino-acid.	I	II	Average.
	per cent	*per cent*	*per cent*
Cystine	1.18	1.18	1.18
Arginine	2.74	2.89	2.82
Histidine	2.40	2.14	2.27
Lysine	0.77	1.01	0.89

Determination of the Free Amino Nitrogen in Hordein.—The solutions used for these determinations were prepared by dissolving 2 gm. of hordein by triturating with 25 cc. of 1 per cent acetic acid in a mortar. Diphenyl ether was used to prevent foaming and a correction made for the gas evolved by the reagents. The large deaminizing bulb of the Van Slyke apparatus was used and the nitrogen evolved measured in the micro-burette. The results are recorded in Table III.

TABLE III.

Free Amino Nitrogen of Hordein Compared with the Lysine Nitrogen.

Total N in sample analyzed.	N gas in sample analyzed.	Pressure.	Temperature.	Amino N in sample analyzed.	Ratio of amino N to total N.	Average ratio of amino N to total N.	One-half lysine N by Van Slyke method.
mg.	*cc.*	*mm.*	*°C.*	*mg.*	*per cent*	*per cent*	*per cent*
18.56	0.20	760	27	0.110	0.59	0.58	0.58
92.80	0.95	760	27	0.522	0.56		

SUMMARY.

1. The basic amino-acids of hordein have been determined by the method of Van Slyke.

2. The analyses indicate that hordein contains about 1 per cent of lysine which has hitherto not been shown to be present in this protein.

3. The free amino nitrogen in hordein has been determined and has been found to be equal to one-half of the lysine nitrogen as determined by the Van Slyke method.

4. The percentages of the different basic amino-acids in hordein are almost the same as those found in gliadin of wheat.

GAS TENSIONS IN THE TISSUES OF THE MOUTH.*

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(Received for publication, March 26, 1919.)

It is important in many problems to know the tensions of gases and volatile substances in the blood and tissues; *e.g.*, oxygen, CO_2, nitrogen ether, CO, etc. It occurred to us that a simple method of determining these tensions would be afforded by filling one of the body cavities or a subcutaneous connective tissue space with air or some other gas mixture. Diffusion must sooner or later establish gaseous equilibrium with the surrounding tissues; the composition of the gas in the cavity can then be determined by analysis.

The most easily available cavity is the mouth. By holding the cheeks blown out and breathing through the nose, which can be done for 15 or 20 minutes at a time, a closed cavity is obtained, for if the mouth were not shut off from the pharynx by the soft palate and tongue the cheeks would not remain distended. To fill the mouth with alveolar air it is necessary merely to make a deep expiration of which the last portion is retained in the mouth. To fill it with air of more than alveolar CO_2 tension, the breath is held for 20 or 30 seconds or longer, a deep expiration is made into a gas sampler,[1] and the cheeks are distended with the last part of the expiration. While this air is being held in the mouth, that in the sampler is analyzed. The air in the mouth is then drawn into the gas analyzer through a fine glass tube inserted between the lips. For initial concentrations of CO_2 higher than the body can

* The expenses of these investigations were defrayed in part by the Loomis Medical Research Fund.

[1] Henderson, Y., and Morriss, W. H., *J. Biol. Chem.*, 1917, xxxi, 221, Fig. 2.

produce the gas mixture is made in a bag and a sample analyzed before the mouth is filled.

The data which we obtained in this way regarding CO_2 are given in Table I.

From these observations it appears that the point of equilibrium for CO_2 in the mouth is about 7.5 per cent, or 54 mm. mercury. As saliva is rich in carbon dioxide it might perhaps tend to make this figure higher than that which would be correct for the tissues.

TABLE I.

Initial concentration of CO_2.	Time held in mouth.	Final tension of CO_2.
per cent	*min.*	*per cent*
Air.	11	2.64
"	11	2.65
5.7	5	6.3
6.9	10	7.3
7.65	4	7.65
7.65	8	7.4
7.8	10	7.55
10.8	4	10.3

In a few observations we have found the tension of CO_2 in saliva to be about 58 mm. of mercury and the content about 7 volumes per cent when determined without the addition of acid. The error from the CO_2 in the saliva is therefore probably inconsiderable.

Recent papers from this laboratory have shown that when the breath is held to the breaking point[2] or air is rebreathed[3] until the CO_2 ceases to rise, a CO_2 tension of about 7.5 per cent is reached. It was pointed out that this is probably the CO_2 tension in the tissues, while that in the venous blood is about 6.6 per cent or 47.5 mm. For determining the oxygen equilibrium various mixtures of air and nitrogen were made. The data are given in Table II.

[2] Henderson, Y., and Prince, A. L., *J. Biol. Chem.*, 1917, xxxii, 325.
[3] Laurens, H., *Am. J. Physiol.*, 1918, xlvi, 147.

TABLE II.

Initial concentration of oxygen.	Time held in mouth.	Final tension of oxygen.
per cent	*min.*	*per cent*
12.90	6	12.15
12.43	11	11.69
11.12	5	10.70
9.85	4	9.25
9.28	10	8.78
9.26	3	9.00
7.57	10	7.25
7.44	4	7.29
6.80	6	6.67
6.55	10	6.07
5.48	10	4.97
2.72	10	2.45
1.79	10	1.65
1.32	11	1.32
0.53	11	0.50
0.06	11	0.05

These figures seem to justify the conclusion that the point of oxygen equilibrium is not above, but possibly is considerably below 7 per cent or 50 mm. of mercury. The rate of diffusion of oxygen is much slower than that of CO_2 and the lower figures in Column 3 are therefore only approximate.[4]

[4] Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1919, xxxviii, 71.

GAS TENSIONS OF THE ABDOMINAL CAVITY, WITH SOME EVIDENCE ON THE DIFFUSION OF GASES WITHIN THE BODY.*

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(Received for publication, March 26, 1919.)

The determination of the alveolar CO_2 in animals (dogs) is liable to considerable error, particularly in those conditions in which the gas tensions of the blood may be of especial significance. Still less can one judge of the oxygen supply to the tissues from analyses of alveolar air, or even of arterial blood.

We find that when air, or other gas mixture, is injected into the abdominal cavity, it comes after a time to have a tension of CO_2 equivalent to that of the arterial blood and alveolar air.

The diffusion of oxygen is much less rapid; owing to this fact and to the form of the oxyhemoglobin dissociation curve, the oxygen consumption by the peritoneum reduces the oxygen tension to 50 ± 5 mm.[1]

The procedure was as follows: A definite amount of gas, usually 1 liter of air or nitrogen, was introduced by means of a hypodermic needle inserted through the abdominal wall in a lower quadrant. At intervals samples were withdrawn and analyzed by the apparatus previously described.[2] In no case has any injury to the peritoneum been apparent when the animal was later killed and autopsied. When blood samples were drawn for analysis, the femoral

* The greater part of this work was done under the War Gas Investigations of the Bureau of Mines and the Chemical Warfare Service, U. S. Army.

The expenses of these investigations were defrayed in part by the Loomis Medical Research Fund.

[1] Henderson, Y., and Stehle, R. L., *J. Biol. Chem.*, xxxviii, 67.

[2] Henderson, Y., *J. Biol. Chem.*, 1918, xxxiii, 32.

artery on one side and the femoral vein on the other were exposed under local anesthesia. The venous blood was drawn by means of a hypodermic syringe without obstructing the blood flow in the vessel.

A general anesthetic was unnecessary and was not used, as the operation causes no discomfort. General anesthesia exerts so powerful an influence upon respiration and the blood gases as seriously to impair the validity of experiments on these functions. Pain, fear, or excitement likewise exert extremely disturbing effects and must be avoided absolutely. With care and gentleness, however, it is possible, and indeed easy, to carry out the exposure and opening of blood vessels and other operations under local anesthesia, without appreciable disturbance of either the physiological or psychological equilibrium of the animal.

Each of the following experiments is one of several in which nearly identical results were obtained. All the time intervals were noted from a clock which was set at 12 at the moment when the gas was injected into the abdomen. The alveolar air samples were taken by the Higgins-Plesch method by means of a mask and bag; the animal rebreathed 200 cc. of air for 30, 40, and 50 seconds. From these three analyses the two which agreed most nearly were averaged to obtain the percentages recorded in the protocols.

Every experiment here reported has been repeated at least once with results essentially identical with those of the protocol selected for publication.

Experiment 1.—Dog, female, 9 kilos.

Time.	Alveolar CO_2 (mm. of mercury).	Incarcerated gases.	
		CO_2 (mm. of mercury).	Oxygen (mm. of mercury).
11.25	38		
12.00			
1 liter of air introduced into abdominal cavity.			
12.30	35	24	
12.50	37	36	126
2.05	39	38	119
2.35	38	39	108
4.35	38	38	90

Experiment 2.—Dog, male, 16.5 kilos.

Time.	Alveolar CO_2 (mm. of mercury).	Incarcerated gases.	
		CO_2 (mm. of mercury).	Oxygen (mm. of mercury).
hrs.			
0			
1 liter of air introduced into abdominal cavity.			
7	38	38	66
30	37	39	53
48	36	34	52
54		37	56

Experiment 3.—Dog, female, 8 kilos.

Time.	Alveolar CO_2 (mm. of mercury).	Incarcerated gases.	
		CO_2 (mm. of mercury).	Oxygen (mm. of mercury).
11.55	41		
12.00			
1 liter of nitrogen introduced into abdominal cavity.			
12.10	39	13	10
12.20		19	14
12.30	40	31	20
12.40		39	26
12.50	42	39	31
1.00		40	39
1.10	39	38	42
1.20		38	44
1.30	41	40	45
2.30	40	39	46
3.30	41	40	45
4.30		38	46

Analyses to determine the oxygen content were made on three samples of this animal's blood. (The animal had an unusually small amount of hemoglobin, a fact without significance in this connection.)

	Oxygen vol. per cent
(*a*) Arterial blood (alveolar air O_2 95 mm , CO_2 40 mm.)....	12.2
(*b*) Blood equilibrated at 39°C. with room air + 5.55 per cent CO_2, *i.e.*, O_2 145 mm. + CO_2 40 mm.....	12.6
(*c*) Blood equilibrated at 39°C. with air drawn from the abdomen, *i.e.*, O_2 46 mm. + CO_2 40 mm.....	10.1
Difference between (*a*) and (*c*) (corresponding to a difference of oxygen tension of 95−46 = 49 mm.).....	2.1

Evidently a great fall of oxygen tension (49 mm.) is necessary before the blood gives up even a small amount (2.1 volumes per cent) of oxygen to the tissues. This fact, with the slow diffusion of oxygen and the consumption by the tissues, explains the low oxygen tension of the abdominal cavity. It probably approximates the local venous tension.

Experiment 4.—Dog, male, 16 kilos.

Time.	Alveolar CO_2 (mm. of mercury).	Incarcerated gas CO_2 (mm. of mercury).
11.45	37	
12.00		
1 liter of air introduced into abdominal cavity.		
12.30	36	6
1.00	38	8
1.30	37	10
2.00		11
3.00	39	17
4.00	38	22
5.00		28
6.00	37	32
7.00		35

The rate of diffusion between air and blood in this experiment was unusually slow. When this dog was killed and autopsied, a thick fat omentum was found covering the whole of the anterior visceral surface and preventing direct contact with the incarcerated air.

From these experiments it appears that when either air or nitrogen is used the equilibrium of CO_2 is reached within 1 hour. The tension of CO_2 in the abdominal air becomes the same as that of the alveolar air. The oxygen equilibrium is reached after an hour and a half when nitrogen is injected, but only after a much

longer period when air is used. Owing to the comparatively slow diffusion of oxygen through the tissues, the equilibrium tension of oxygen in the abdominal air is much below that of the alveolar air and arterial blood. It may approximate the local venous tension (cf. Experiment 3). The impediment offered by a large fat omentum is illustrated in Experiment 4; animals of this rare type, in which a constant tension of CO_2 is not attained in 1 hour, should be discarded.

Experiment 5 demonstrates that the CO_2 tension of the incarcerated air comes into equilibrium with that of the arterial blood.

Experiment 5.—Dog, male, 12 kilos. 1 liter of air was injected into the abdomen. 2 hours later analyses for CO_2 were made on the alveolar and abdominal airs. Tensions of 43 and 42 mm. respectively were found. Analyses for CO_2 were then made on the arterial and venous blood and on 3 samples of blood equilibrated at 39°C. to air containing (*a*) 22, (*b*) 40, and (*c*) 58 mm. of CO_2. The analyses showed the CO_2 contents of these bloods to be in volumes per cent: arterial 38, venous 43, sample (*a*) 26, (*b*) 34, and (*c*) 44.

By plotting the CO_2 dissociation curve of the blood as defined by (*a*), (*b*), and (*c*), and noting on this curve the point corresponding to the CO_2 content of the arterial blood, a tension of 44 mm. is found, showing that the tension of CO_2 in the arterial blood was identical with that of the alveolar and abdominal air.

Experiment 6, on artificial acidosis, shows how closely changes of arterial CO_2 tension are followed by the abdominal air (cf. Columns 2 and 8 in the protocol).

Experiment 6.—Dog, male, 12 kilos. Procedure and analyses were the same as in Experiment 5. The blood Samples, (*a*), (*b*), and (*c*) were equilibrated at 37°C. to air containing 17, 40, and 60 mm. of CO_2 respectively, and analyzed; from these data the dissociation curves were plotted; the points in the curves corresponding to the CO_2 content of the arterial blood were then noted, and the tensions of CO_2 taken from the abscissæ of these points (Column 8).

Time.	Abdominal air tension of CO_2 (mm. of mercury).	Blood CO_2 analyses.					Indicated arterial CO_2 tension (mm. of mercury).
		Arterial.	Venous.	Sample (a).	Sample (b).	Sample (c).	
		vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	
12.00							
Injected 2 liters of air into abdomen.							
12.15	33						
1.35	41						
1.45	40	42	44	25	40	47	41
2.15							
Injected 10 cc. N HCl intravenously, 2 cc. per min.							
2.45	22	24					
3.25	37						
3.45	37	37	45	25	38	44	37
5.05							
Injected 36 cc. N HCl intravenously, 2 cc. per min.							
5.45	16	19	33	20	31	33	16
6.40	19	23	38	21	32	37	20

These data will be plotted in a figure to be published in a later paper on the CO_2 dissociation curves of the blood in experimental acidosis and some allied conditions. It is of interest to note in this experiment the correlation of the reduction of the CO_2 capacity of the blood and the lowering of the alveolar CO_2 tension; also the increasing difference between the CO_2 content of the arterial and venous blood, indicating a slowing of the circulation.

A criticism may be made of some of the data in Experiment 6 on the ground that, as found by some investigators,[3-5] the CO_2 capacity of blood is influenced by variations in the oxygen tensions to which it is simultaneously exposed. In observations on

[3] For older literature see Zuntz, N., in Hermann, L., Handb. Physiol., Leipsic, 1882, iv, pt. 2, 81.

[4] Christiansen, J., Douglas, C. G., and Haldane, J. S., *J. Physiol.*, 1914, xlvii, 244.

[5] Parsons, T. R., *J. Physiol.*, 1917, li, 440.

this point, to be presented in a later paper, we have found that in whole blood the effect of pure nitrogen as compared with air on CO_2 capacity is barely appreciable. The effect on defibrinated blood,—which other investigators have used,—is much greater. Deoxygenation with hydrogen exerts a still greater influence upon the CO_2 capacity of the blood.

In repeated experiments it was found that when gas mixtures rich in CO_2 were injected into the abdomen the CO_2 was rapidly absorbed and eliminated through the lungs.

In several experiments also pure oxygen was injected into the abdomen, while the animal was rebreathing a small volume of air in a spirometer through a cartridge of alkali. Under these con ditions CO_2 elimination from the lungs was not interfered with, while the peritoneum was the only avenue of entrance for oxygen into the body. In some experiments life continued under these conditions for several hours; the tension of oxygen in the re-breathed air fluctuated around 4 per cent. When the spirometer was filled with nitrogen, oxygen diffused outward from the lungs.

Experiment 7.—Dog, male, 12 kilos. 1 liter of nitrogen was injected into the abdomen. 5 hours later the animal was killed by a blow on the head. The abdominal gas was analyzed before death and at intervals for 18 hours thereafter.

	Incarcerated gases.	
	CO_2 (mm. of mercury).	Oxygen (mm. of mercury).
Before death	38	50
30 min. after death	49	47
1 hr. " "	56	45
18 hrs. " "	188	22

Experiment 7 shows that after death the consumption of any available oxygen, and the production, or liberation, of a much more than proportionate amount of CO_2 continue for at least 18 hours.

Mention may be made here of the fact that under etherization (without morphine) we have found the tension of ether vapor in the abdominal air (by the use of a Haldane gas analyzer, with a

specially prepared paraffin oil as the absorbent) to be about 4 per cent or 29 mm. Boothby[6] found a tension of 51 mm. necessary for full anesthesia in man, and thinks the figure 31 mm. which he calculates as that representing the estimate of Waller,[7] and his coworkers to be too low. Our observation, however, is practically identical with that found by the latter investigators.

In experiments on carbon monoxide, in which the tension of CO in the inspired air was 0.1 per cent, distinct indications of this gas were found after 1 hour in the abdominal air.

After the foregoing observations were made we noticed Zuntz' article[8] on the blood gases and found there that observations on our general topic have been made previously by Davy[9] in 1823, by Leconte and Demarquay[10] in 1859, and by others. (This topic was once of interest in relation to the ancient belief that exposure of a tissue to air was a cause of inflammation.) In general the data of these investigations, allowing for some obvious inaccuracies due doubtless to crude analytical methods, are substantiated by our findings.

CONCLUSIONS.

The contribution which we make consists in the demonstration that the CO_2 tension of the abdominal air soon becomes equivalent to that of the pulmonary alveolar air and of the arterial blood, and that under abnormal conditions (experimental acidosis) in which it is not easy or safe to cause the rebreathing necessary to obtain an alveolar sample, the CO_2 tension in the abdominal air falls in close correspondence to the arterial CO_2 tension.

Diffusion of oxygen through the tissues is much slower than is that of CO_2. The tension of oxygen in the abdominal air at equi-

[6] Boothby, W. M., *J. Pharmacol. and Exp. Therap.*, 1913–14, v, 379.

[7] Waller, A. D., Hewitt, F., Blumfeld, Gardner, J. A., and Buckmaster, G. A., Anaesthetics, Brit. Association for the Advancement of Science, London, 1911.

[8] Zuntz, N., in Hermann, L., Handb. Physiol., Leipsic, 1882, iv, pt. 2. 59.

[9] Davy, J., Phil. Tr., 1823, 496.

[10] Leconte, C., and Demarquay, J., *Arch. gén. méd.*, 1859, xiv, 111, 424, 545.

librium is about 45 mm. of mercury, much below that of either the arterial or venous blood and corresponding therefore probably to that of the tissues.

When the air breathed contains carbon monoxide this gas appears in the abdominal air.

We find the tension of ether vapor in the abdominal air during anesthesia to be 29 mm. mercury.

A SYSTEM OF BLOOD ANALYSIS.

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(Received for publication, March 29, 1919.)

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INTRODUCTION.

The main purpose of the research recorded in this paper has been to combine a number of different analytical procedures into a compact system of blood analysis, the starting point for which should be a protein-free blood filtrate suitable for the largest possible number of different determinations. It need scarcely be pointed out what a convenience and advantage it would be if one could take the whole of a given sample of blood and at once prepare from it a protein-free blood filtrate suitable for the determination of all or nearly all the water-soluble constituents, non-protein nitrogen, urea, creatinine, creatine, uric acid, and sugar.

In connection with our work on the problem we have also had in mind the desirability of reducing as far as practicable the amount of blood filtrate to be used for each determination, for by means of such reduction the total usefulness of the filtrate is correspondingly increased. There is no hard and fast limit as to the extent to which this reduction can be carried. It is doubtful, however, whether it is sound analytical practice regularly to use the smallest possible amount of material for each determination; whether, for example, blood filtrates corresponding to only 0.1 cc.

of blood should regularly be used for non-protein nitrogen determinations, because it may sometimes be advantageous or necessary to take no more. In this paper we deal chiefly with a semi microchemical scale of work representing only a moderate reduction of the quantities ordinarily taken for colorimetric work with the 60 mm. Duboscq colorimeter.

One of the main obstacles encountered in attempts to develop a definite system of blood analysis of the kind we have had in mind has been the determination of the uric acid. For several years we have had serious doubts as to the full trustworthiness of the uric acid results heretofore recorded in the literature; moreover, to be reasonably accurate the determination has required more blood (about 25 cc.) than can be obtained except in isolated special cases. A large share of the work involved in this research has therefore been a critical study of the uric acid determination; and a modification of the Folin-Denis-Benedict method has been developed which requires the filtrate from only 2 cc. of blood, and which we believe to be more dependable as well as more simple and convenient than the original method.

We have also satisfactorily solved the problem of how to make and keep standard uric acid solutions, and we have devised a new colorimetric method for the determination of sugar in blood.

The determinations included in this research, namely non-protein nitrogen, urea, creatinine, creatine, uric acid, and sugar, can all be determined in the filtrate obtained from 10 cc. of blood.

Preparation of Protein-Free Blood Filtrates.

The pivotal point in our projected general scheme of blood analysis was necessarily a searching review of the most promising methods which have been used for precipitating the blood proteins in connection with the various analytical procedures in common use. As a working principle or guide in this search we have first of all required that the procedure employed must permit the quantitative recovery of at least 10 mg. of uric acid and creatinine when added to 100 cc. of sheep, beef, or chicken blood, and that the total non-protein nitrogen must certainly be no higher than the figures obtained from a corresponding trichloroacetic acid filtrate representing a 10 per cent trichloroacetic acid concentration (in

the diluted unfiltered blood mixture)—or a corresponding 1.5 per cent *m*-phosphoric acid filtrate.

While we are not dependent on the urease method for the determination of the urea, we have, nevertheless, deemed it imperative that the blood filtrate must also be of such a character as readily to permit the use of the urease method for the determination of this important constituent. We do not claim to have exhausted this line of inquiry, for it is a laborious process to determine the merits and shortcomings of any particular reagent in connection with such a comprehensive program. None of the precipitation procedures described in recent years is free from serious shortcomings. Kahlbaum's phosphotungstic acid or sodium phosphotungstate (prepared by ourselves) met our requirements when used under certain very definite conditions, and for a time we concentrated our efforts on the standardization of these reagents and on the adaptation of the various analytical procedures to the blood filtrates obtained from them.

In connection with our work on sodium phosphotungstate we have discovered a new protein precipitant which probably has never before been used in blood analysis. We refer to it as new protein precipitant because so far as we have been able to learn it has never before been used in that capacity. This substance is tungstic acid. Tungstic acid, like sodium phosphotungstate or phosphotungstic acid, must be used in a definite way, but the necessary conditions are not difficult to find. Less than 1 gm. is used for the precipitation of the proteins from 10 cc. of blood, yet the precipitation is more complete than that produced by 10 gm. of trichloroacetic acid, and the filtrate obtained gives no trouble in connection with any of the determinatives so far investigated. Neither creatinine nor uric acid is carried down by the precipitate within the conditions to be described. As much as 20 mg. of uric acid may be added to 100 cc. of blood without incurring any loss by absorption.

The blood protein precipitation obtained by the help of tungstic acid is interesting. The precipitation is completed within a few seconds. When the mixture is shaken hard, the sound is almost like that of shaken mercury and the hardest kind of shaking will not produce more than a trace of foam. The precipitate is very fine, yet does not go through good filter paper and does not stop

up the pores. The filtration is slow, but the total amount of filtrate obtained is nearly as large as that obtained with trichloroacetic acid. If the precipitated mixture is heated in a water-bath for 2 or 3 minutes, the precipitate settles spontaneously. With this modification, centrifuging can be substituted for the filtration as the supernatant liquid is water-clear and contains no more nitrogen than the unheated filtrate. For the present we do not care to recommend this process except for quantities of blood so small that one cannot afford to filter. The statement of Folin and Denis that no precipitation involving the use of heat is permissible is probably erroneous as has been pointed out by Bock, although it is true for the *m*-phosphoric acid precipitation and probably for many others. Unless some compelling advantage is gained by the use of heat, precipitation in the cold does seem to be the safer process.

There are many other points of interest to be investigated in connection with tungstic acid as a precipitant, but as these have no direct bearing on the problem of this research, further consideration of them here is omitted. As a precipitant for blood proteins we believe that tungstic acid will prove more useful than any other reagent yet proposed.

The precipitation of the blood proteins by means of our new reagent is made in the following manner. Transfer a measured amount of blood into a flask having a capacity of fifteen to twenty times that of the volume taken. Dilute the blood with 7 volumes of water and mix. With an appropriate pipette add 1 volume of 10 per cent solution of sodium tungstate ($Na_2WO_42H_2O$) and mix. With another suitable pipette add to the contents in the flask (with shaking) 1 volume of $\frac{2}{3}$ normal sulfuric acid. Close the mouth of the flask with a rubber stopper and give a few vigorous shakes. If the conditions are right hardly a single air bubble will form as a result of the shaking. Much oxalate or citrate interferes with the coagulation and later with the uric acid determination. 20 mg. of potassium oxalate is ample for 10 cc. of blood. Citrate, except in the minimum amount, is to be avoided. When a blood is properly coagulated, the color of the coagulum gradually changes from pink to dark brown. If this change does not occur, the coagulation is incomplete, due, in every case we have encountered, to too much oxalate or citrate. In such an

emergency the sample may be saved by adding 2 normal sulfuric acid drop by drop, shaking vigorously after each addition and allowing the mixture to stand for a few minutes before adding more, until the coagulation is complete. Pour the mixture on a filter large enough to hold the entire contents of the flask and cover with a watch-glass. If the filtration is begun by pouring the first few cc. of the mixture down the double portion of the filter paper and withholding the remainder till the whole filter has been wet, the filtrates are almost invariably as clear as water from the first drop. If a filtrate is not perfectly clear, the first 2 or 3 cc. may have to be returned to the funnel. (Filter papers of the following diameters will meet all ordinary needs: 11, 12½, 15, and 18½ cm.)

It will be noted that the precipitation of the blood proteins is not made in volumetric flasks. Our procedure is adapted to the full use of practically all of a given sample of blood, for by this system 7, 9, or 12 cc. can be utilized just as well as 5 or 10. For this work we have devised a special blood pipette,[1] a sketch of which is given in Fig. 1. This is simply a 15 cc. pipette, graduated from the long tip into 1 cc. portions. The lower part is more or less like that of a volumetric pipette, thus permitting one to draw the blood directly from small, narrow bottles. We find it convenient to use three such pipettes; one for the blood, one for the sodium tungstate solution, and one for the sulfuric acid. The water used for diluting the blood may be measured with a cylinder.

The preparation of protein-free blood filtrates by this new process is so simple that no one need go astray, provided that the sodium tungstate and the ⅔ normal sulfuric acid are correct. The only doubtful point is the quality of the sodium tungstate used. The acid is intended to set free the whole of tungstic acid with about 10 per cent excess (and to neutralize the carbonate usually present in commercial tungstates). A

[1] The pipettes are made for us by the Emil Greiner Co., New York.

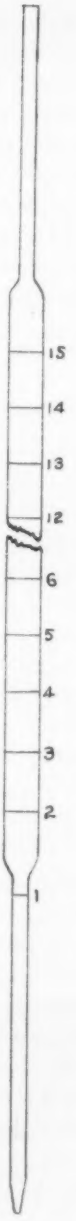

FIG. 1.

greater excess of sulfuric acid must not be used, for if this is the case a large part of the uric acid will be lost. A safe and convenient criterion is to test the blood filtrate obtained with Congo red paper. The reaction should be negative or at the most just perceptible. We have employed three different tungstates, and all worked equally well. The product we now use was obtained from the Primos Chemical Company, Primos, Pa.

The carbonate content of sodium tungstate is easily determined as follows: To 10 cc. of 10 per cent solution, add one drop of phenolphthalein and titrate with 0.1 normal hydrochloric acid. Each cc. of hydrochloric acid corresponds to 1.06 per cent of sodium carbonate. The amount of acid required for the titration should not exceed 0.4 cc.

TABLE I.

Comparison of Non-Protein Nitrogen in Blood Filtrates Obtained by Means of Trichloroacetic Acid and Tungstic Acid.

Source.	Mg. per 100 cc. blood.	
	Trichloroacetic acid.	Tungstic acid.
Human	35.7	31.5
"	32.4	28.2
"	35.4	33.0
"	42.0	42.0
"	50.7	48.6
Chicken	66.5	54.5
"	48.0	42.5
"	53.2	44.4
"	50.0	44.8
"	51.6	47.6

Our blood filtrates are nearly neutral, 10 cc. of filtrate requiring only about 0.2 cc. of 0.1 normal sodium hydroxide when titrated with phenolphthalein as indicator. If the filtrates are to be kept for any length of time, more than 2 or 3 days, they need some preservation. One or two drops of toluene or xylene is adequate for the filtrate obtained from 10 cc. of blood. Xylene seems to be fully as effective as toluene as a preservative.

The precipitation process just described works equally well with any kind of blood which we have yet tried—human, beef, sheep,

chicken, dog, and rabbit—and numerous comparisons with the trichloroacetic acid precipitation have shown that the non-protein nitrogen obtained by the process invariably tends to be lower than the figures given by trichloroacetic acid. The figures of Table I illustrate this point.

Determination of Non-protein Nitrogen.

The protein-free blood filtrates prepared by our new process lend themselves perfectly to nitrogen determinations by the direct Nesslerization process of Folin and Denis.[2] As a result of further experience with that method we are able to introduce certain modifications believed to represent improvements.

The acid digestion mixture is made as follows: Mix 300 cc. of phosphoric acid syrup (about 85 per cent H_3PO_4) with 100 cc. of concentrated sulfuric acid. Transfer to a tall cylinder, cover well to exclude the absorption of ammonia, and set aside for sedimentation of calcium sulfate. This sedimentation is very slow, but in the course of a week or so the top part is clear and 50 to 100 cc. can be removed by means of a pipette. (It is not absolutely necessary that the calcium should be thus removed, but it is probably a little safer to have it done.) To 100 cc. of the clear acid add 10 cc. of 6 per cent copper sulfate solution and 100 cc. of water. 2 cc. of this solution are substantially equivalent to 1 cc. of the acid mixture previously described by Folin and Denis. We prefer this diluted acid, first, because the objectionable viscosity of the undiluted reagent is practically eliminated, and, second, because we now use for a nitrogen determination only 5 cc. (instead of 10 cc.) of blood filtrate, and 1 cc. of acid (corresponding to 0.5 cc. of the undiluted acid reagent).

The micro-Kjeldahl digestion is made as before in test-tubes. While we still have an abundant supply of Jena test-tubes we no longer use them for this digestion because the Pyrex ignition test-tubes are very much better in nearly every respect. Test-tubes having a capacity of about 75 cc. (200 × 25 mm.) are suitable for this purpose, and if made of Pyrex ignition glass are almost as good as those of pure silica. These test-tubes should be graduated

[2] Folin, O., and Denis, W., Nitrogen determination by direct Nesslerization, *J. Biol. Chem.*, 1916, xxvi, 473.

at 35 cc. and at 50 cc. on two sides, or by means of diamond marks going entirely around. The reason for this graduation is that we now Nesslerize in the digestion tube.

In micro-Kjeldahl digestions severe bumping is much more common than in ordinary macro-Kjeldahl digestions, but even in the latter the bumping phenomenon is often a source of serious difficulties. Glass beads, pumice stone, pieces of porcelain, etc., are used to remedy this trouble. For years ordinary quartz pebbles have been used in this laboratory, but at times these too have failed to prevent loss of a determination through sudden violent bumping. Occasionally a pebble may be hurled out of a 200 mm. test-tube by one intensive explosion. At other times no trouble at all is encountered. Langstroth,[3] who seems to have encountered very severe and persistent bumping, resorts to the device of holding the test-tube in as nearly a horizontal position as the contents in the tube will permit; but since it takes half an hour to boil off the liquid which ordinarily can be boiled off in less than 10 minutes, that remedy cannot be considered satisfactory. Langstroth seems to have concluded that such bumping is peculiar to blood filtrates obtained by means of *m*-phosphoric acid, but the phenomenon is quite general. Prolonged boiling of pure water in any glass vessel will lead to the most intense bumping. The most important cause of bumping is certainly the condition of the test-tube. A new test-tube does not cause bumping, but if the same one is used over and over again in one session, the bumping becomes progressively worse. The worst kind of a test-tube (or Kjeldahl flask) can be made as good as a new one by thoroughly drying it; also by rinsing with alcohol.

The reason why dry test-tubes cause less bumping and why dry pebbles tend to prevent bumping is manifestly the presence of very fine pores filled with air in the test-tube and in the pebbles. Until this air has been driven out by heat, localized formation of steam occurs and the boiling is smooth and even, but as these pores are gradually filled with the liquid the bumping begins. By keeping on hand a sufficiently large number of dry test-tubes so that no one need be used more than twice in one session the bumping phenomenon, in the presence of a fresh (that is a *dry*) quartz pebble or piece of granite, is almost entirely eliminated.

[3] Langstroth, L., Notes on Folin's direct Nesslerization method for the determination of nitrogen, *J. Biol. Chem.*, 1918, xxxvi, 377.

The Nesslerization process has also been simplified. The preliminary neutralization of the acid has been eliminated. The Nessler solution which we now use for all Nesslerizations is made as follows: The stock solution of mercuric potassium iodide can be made just as previously described. Dissolve 150 gm. of potassium iodide in 100 cc. of warm water, add 200 gm. of mercuric iodide, stir until the latter is dissolved, and dilute to a volume of about 1 liter; filter, if necessary, and dilute to a final volume of 2 liters. It is advantageous to make a large volume of this solution for a second sediment may form which takes a long time to settle.

The mercuric iodide obtainable from dealers frequently contains insoluble impurities (probably mercuric sulfide and mercurous iodide) which make it difficult to obtain a clear solution by the addition of potassium iodide. In such cases it is advisable to let the dissolved double iodide stand for 1 or 2 days and then filter, before diluting to volume.

Because of the difficulties encountered in obtaining high grade mercuric iodide, we have devised a new process for making the mercuric potassium iodide solution. This process is as follows: Transfer 150 gm. of potassium iodide and 110 gm. of iodine to a 500 cc. Florence flask; add 100 cc. of water and an excess of metallic mercury, 140 to 150 gm. Shake the flask continuously and vigorously for 7 to 15 minutes or until the dissolved iodine has nearly disappeared. The solution becomes quite hot. When the red iodine solution has begun to become visibly pale, though still red, cool in running water and continue the shaking until the reddish color of the iodine has been replaced by the greenish color of the double iodide. This whole operation usually does not take more than 15 minutes. Now separate the solution from the surplus mercury by decantation and washing with liberal quantities of distilled water. Dilute the solution and washings to a volume of 2 liters. If the cooling is begun in time, the resulting reagent is clear enough for immediate dilution with 10 per cent alkali and water, and the finished solution can at once be used for Nesslerizations.

The cost of the chemicals called for in this process of making Nessler's solution is less than when starting with mercuric iodide, and the disagreeable impurities present in many samples of mercuric iodide are avoided.

From the stock solution of mercuric potassium iodide, made according to either of the processes described above, we prepare the final Nessler solution as follows: From completely saturated caustic soda solution containing about 55 gm. of NaOH per 100 cc. decant the clear supernatant liquid and dilute to a concentration of 10 per cent. (It is worth while to determine by titration that a 10 per cent solution has been obtained within an error of not over 5 per cent.) Introduce into a large bottle 3,500 cc. of 10 per cent sodium hydroxide solution, add 750 cc. of the double iodide solution and 750 cc. of distilled water, giving 5 liters of Nessler's solution.

The Nessler solution so obtained contains enough alkali in 15 cc. to neutralize 1 cc. of the diluted phosphoric-sulfuric acid mixture and to give a suitable degree of alkalinity for the development of the color given by ammonia at a volume of 50 cc.

(In other Nesslerizations, as in urine analysis when there is no acid to be neutralized, 10 cc. of the Nessler reagent per 100 cc. of Nesslerized ammonia solution is the correct amount.)

Concise Description of Non-Protein Nitrogen Determination.—Introduce 5 cc. of the protein-free blood filtrate into a dry 75 cc. test-tube graduated at 35 cc. and at 50 cc. Add 1 cc. of the sulfuric-phosphoric acid mixture described on page 87. Add a dry quartz pebble and boil vigorously over a microburner until the characteristic dense acid fumes begin to fill the test-tube. This is usually accomplished in from 3 to 7 minutes. When the fumes are unmistakable, cut down the size of the flame so that the contents of the tube are just visibly boiling, and close the mouth of the test-tube with a watch-glass or a very small Erlenmeyer flask. Continue the heating very gently for 2 minutes from the time the fumes began to be unmistakable, even if the solution has become clear and colorless at the end of 20 to 40 seconds. If the oxidations are not visibly finished at the end of 2 minutes the heating must be continued until the solution is nearly colorless. Such cases are very rare; the oxidation is almost invariably finished within the 1st minute. Allow the contents to cool for 70 to 90 seconds and then add 15 to 25 cc. of water. Cool further, approximately to room temperature, and add water to the 35 cc. mark. Add, preferably with a pipette, 15 cc. of the Nessler solution described above. Insert a clean rubber stopper and mix. If the solution

is turbid, centrifuge a portion before making the color comparison with the standard. The standard most commonly required is 0.3 mg. of N (in the form of ammonium sulfate) in a 100 cc. flask. Add to it 2 cc. of the sulfuric-phosphoric acid mixture, about 50 cc. of water, and 30 cc. of Nessler solution. Fill to the mark and mix. The unknown and the standard should be Nesslerized at approximately the same time. If the standard is set at 20 mm. for the color comparison, 20 divided by the reading and multiplied by 30 gives the non-protein nitrogen in mg. per 100 cc. of blood.

Determination of Urea.

Investigations on the most satisfactory method for the determination of urea have been pursued for the last 2 or 3 years (partly with the assistance of G. L. Foster and Guy Youngburg). Much of the work done on the subject has been an endeavor to find a direct Nesslerization process without the use of Merck's blood charcoal. Our attempts have not resulted in any thoroughly satisfactory method because very small amounts of ammonia cannot be Nesslerized in the presence of either amino-acids or peptones. Direct Nesslerization, even with the help of charcoal, cannot be made except at the expenditure of more blood filtrate than is actually used in the final stages of the determination, and a strictly economical use of the blood filtrate we have considered a fundamentally important point in our system of blood analysis. Direct Nesslerization has therefore been abandoned in connection with the determination of urea in blood. Extensive use has also been made of the permutit extraction after first decomposing the urea with urease, but this process has proved somewhat fallacious with bloods in which the total urea nitrogen is small, as in many normal bloods, so this process also has been abandoned. Since probably no other determination will be as useful and important to the clinician as the determination of the blood urea, we have considered it of the utmost importance to get a method which is as simple as possible, but above all reliable. In this connection we have had in mind not only the needs of well equipped hospital laboratories, but also the needs of private practitioners.

For the hydrolysis of the urea we make use of jack bean urease, or the autoclave; for the isolation of the ammonia produced we employ aeration or distillation; thus we have four combinations any one of which will give satisfactory results. The autoclave process is, of course, not advantageous for single urea determinations, but on the other hand is distinctly useful when it is a question of a large series of determinations, or when creatine determinations are also to be made, because the hydrolysis of the urea can then be accomplished simultaneously with the conversion of the creatine into creatinine. The chief merit of the autoclave process for decomposing urea in blood filtrates lies perhaps in the fact that by its help one is sure to get all the urea nitrogen; the values obtained may be too high, but not too low. Yet the results obtained by the autoclave process are as a matter of fact usually identical and rarely as much as 1 mg. per 100 cc. of blood higher than those obtained by the urea process.

Urease Decomposition.—For the decomposition of urea by means of urease we use exclusively jack bean powder extracts and not so called purified or concentrated urease preparation. It is doubtless possible to prepare such, but those obtainable in the market are usually less active than an equal weight of jack bean powder, and of course are much more expensive.[4] An excellent urease solution can be prepared from jack bean powder in the following manner: Transfer to a 200 cc. flask or bottle about 3 gm. of permutit powder. Wash this by decantation, once with 2 per cent acetic acid, then twice with water. Add to the moist permutit in the flask 100 cc. of 30 per cent alcohol (35 cc. of 95 per cent alcohol mixed with 70 cc. of water). Then introduce 5 gm. of jack bean meal and shake for 10 minutes. Filter and collect the filtrate in three or four different small clean bottles. Set one aside for immediate use; it will remain serviceable at least 1 week at ordinary room temperature, if not exposed to direct sunlight. Put the others on ice where they will remain good for 3 to 5 weeks. The filtrate contains substantially the whole of the urease present in the jack bean powder and is very active. In the presence of a suitable phosphate mixture, 1 cc. added to 300 mg. of urea nitrogen at a volume of 200 cc. will yield 37 to 42 mg. of urea nitrogen

[4] The Arlington Chemical Co. supplies jack bean meal in a finer state of division than one can readily make by hand.

in 1 hour at 20°C. In 18 hours all the urea will be decomposed. The use of permutit makes the extract free from ammonia (5 cc. containing less than 0.01 mg.), nor does more ammonia develop on standing.

Urease decompositions of urea are never dependable except in the presence of some buffer mixtures by which the reaction of the solution can be kept within certain limits. The action of such mixtures is twofold. They not only accelerate the decomposition of the urea, but also prolong greatly the acting period of the enzyme. When urease solutions prepared as described above are added to urea dissolved in distilled water, it not infrequently happens that the enzyme acts for only a few minutes and then stops altogether, so that no more ammonia is obtained after 24 hours than after 15 minutes. That the enzyme is only dormant and not entirely destroyed is shown by the fact that on adding phosphate mixture to the solution after 24 or even 48 hours standing, renewed urea decomposition begins and then continues for a long time. The *Auxourease* found by Jacobi[5] to be present in blood serum represents probably nothing more or less than a preserving action of amphoteric serum proteins on the urease, action similar to that of phosphates.

In the course of our investigations on the determination of urea in blood filtrates by means of urease, it was accidentally found that other phosphates than those investigated by Van Slyke are equally good or better. When the titratable acidity of *m*-phosphoric acid blood filtrates was neutralized with sodium bicarbonate, the urease action on (added) urea was surprisingly active and long sustained. (The urea content of such blood filtrates can be determined conveniently both by the urease and by the autoclave processes.) In consequence of this discovery, a series of experiments was made with pyro- and *m*-phosphates, and our observations have led to the conclusion that a solution containing 140 gm. of sodium pyrophosphate (U.S.P.) and 20 gm. of glacial phosphoric acid per liter is probably better than any of the phosphate mixtures investigated by Van Slyke. We are at a loss for an explanation, for Van Slyke's mixtures cover the field sufficiently well from the standpoint of hydrogen and hydroxyl ion concentrations.

[5] Neumann, R., Über die Aktivierung der Soja-Urease durch menschliches Serum, *Biochem. Z.*, 1915, lxix, 134.

A thorough study of this subject has not been made, but it appears that the pyrophosphates are less injurious to urease than *o*-phosphates. One experiment may be cited.

Solutions containing mono- and disodium phosphate in the molecular ratios 1:1 and 1:2 were prepared. To 300 mg. of urea nitrogen in 200 cc. flasks were added (1) 5 cc. of phosphate 1:1, (2) 5 cc. of phosphate 1:2, and (3) 5 cc. of the pyrophosphate solution described above. To such mixtures were added water to 200 cc. and 1 cc. of urease solution (temperature 18°C.). Table II shows the results.

TABLE II.

Comparison of Effect of Different Buffer Mixtures on Rate of Hydrolysis of Urea by Action of Urease.

Buffer mixture.	Ammonia N.			
	15 min.	30 min.	1 hr.	19 hrs.
	mg.	*mg.*	*mg.*	*mg.*
Phosphate 1:1	5.7	9.6	21.4	266
" 1:2	5.7	9.5	19.8	180
Pyrophosphate	12.5	20.8	37.6	300

Determination of Urea by Urease Decomposition and Distillation.

Transfer 5 cc. of the tungstic acid blood filtrate to a *clean* and *dry* Pyrex ignition tube (capacity about 75 cc.). The graduated Pyrex tubes recommended for the non-protein nitrogen determination should never be used for urea determinations, because they have contained Nessler solutions and Nessler solutions leave behind films of mercury compounds which destroy the urease. If those tubes must be used, they should first be washed with nitric acid to remove the mercury films. Add to the blood filtrate two drops of the pyrophosphate solution described above or two drops of a molecular *o*-phosphate solution ($\frac{1}{3}$ molecular monosodium phosphate plus $\frac{2}{3}$ molecular disodium phosphate). Then add 0.5 to 1 cc. of the urease solution described on page 92 and immerse the test-tube in a beaker of warm water and leave it there for 5 minutes. The temperature of the water is not very important but should not exceed 55°C. The warm water can perhaps scarcely be said to be essential, for the hydrolysis is very rapid at room

temperature, but we nevertheless much prefer to use it. If no hot water is used, continue the digestion for 10 to 15 minutes, or as much longer as is convenient. The ammonia formed can be conveniently and quickly distilled into 2 cc. of 0.05 normal hydrochloric acid contained in a second test-tube. The second test-tube should not be so heavy as the ordinary test-tubes and should be graduated at 25 cc. A simple and compact arrangement for this distillation is indicated by Fig. 2. The test-tube which serves as a receiver is held in place by means of a rubber stopper in the side of which has been cut a fairly deep notch to permit the escape of air (and some steam). The rubber stopper serving as a holder for the receiver fits quite loosely to the delivery tube by means of which the two test-tubes are connected. The delivery tube must, of course, be so adjusted as to reach below the surface of the hydrochloric acid solution in the receiver before the distillation is begun.[6]

Add to the hydrolyzed blood filtrate a dry pebble, 2 cc. of saturated borax solution, and a drop or two of paraffin oil; insert firmly the rubber stopper carrying both delivery tube and receiver, and boil moderately fast over a microburner for 4 minutes. The size of the flame should never be cut down during the distillation, nor should the boiling be so brisk that the emission of steam from the receiving tube begins before the end of 3 minutes. At the end of 4 minutes slip off the receiver from the rubber stopper and put it in the position shown in Fig. 2. Continue the distillation for 1 more minute and rinse off the lower outside part of the delivery tube with a little water. Cool the distillate with running water, dilute to about 20 cc., and add 2.5 cc. of the Nessler solution described on page 90. Fill to the 25 cc. mark and compare in the colorimeter with a standard containing 0.3 mg. of N in a 100 cc. flask and Nesslerized with 10 cc. of the Nessler solution. The standard and unknown should always be Nesslerized as nearly simultaneously as practicable.

Calculation.—Multiply 20 (the height of the standard in mm.) by 15 and divide by the colorimetric reading to get the urea nitrogen per 100 cc. of blood. The reasons for this calculation are, of course, to be found in the fact that the standard containing 0.3

[6] The distillation apparatus can be obtained from Knott Apparatus Co., Boston.

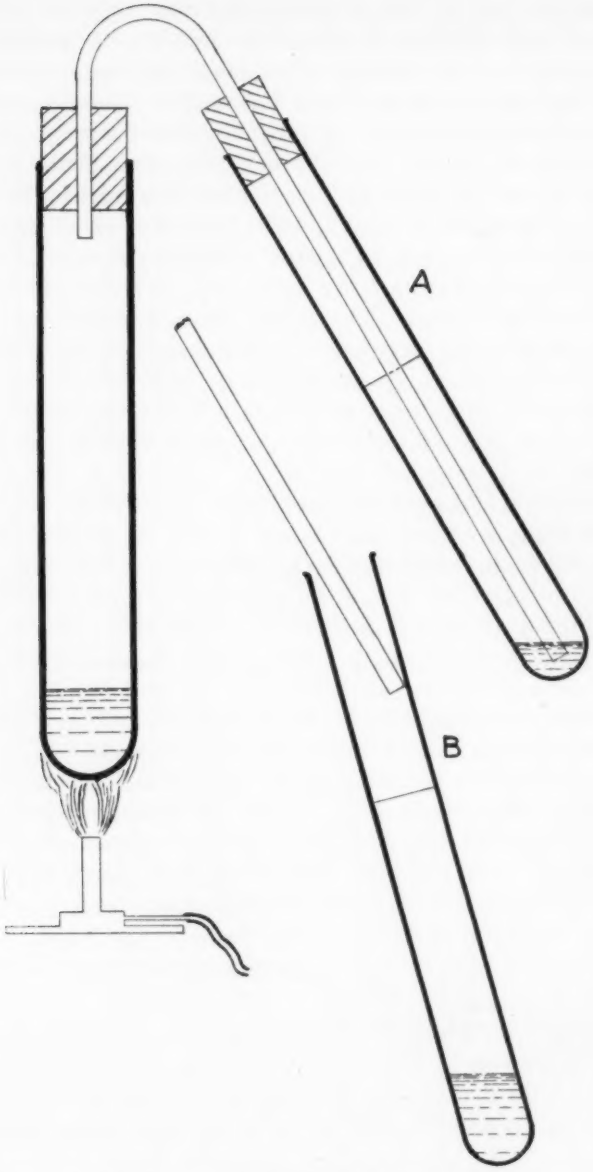

FIG. 2. A, at beginning; B, toward end of distillation.

mg. of N is diluted to 100 cc., while the unknown, which corresponds to 0.5 cc. of blood, is diluted to only 25 cc.

It is even more important in this distillation than in the nonprotein nitrogen digestion that the Pyrex test-tube should not be in a condition that leads to bumping. Dry the tube, or rinse it with alcohol, after each determination.

Borax, the alkali used in this distillation, is strong enough to set free the ammonia, yet is so weak that the blank ammonia which it gives with 5 cc. of urease solution is scarcely any greater than that obtained by the aeration process.

It will be noted that no condenser is used in connection with the microdistillation described above. Since ammonia can be quantitatively recovered by means of an air current, it would seem that it should be recovered as easily by means of a current of steam, especially since the first part of the distillate, containing probably 90 per cent of the ammonia, is automatically condensed just as in ordinary macro-Kjeldahl distillations. A few experiments made along this line indicate that condensers are indeed superfluous even in macro-Kjeldahl distillations.

The other three modifications for the determination of urea in the blood filtrates can be referred to very briefly, for they will be used only by those who are already familiar with the principles and practices involved.

Urea Determination by Means of Urease and Aeration.—The decomposition of the urea is made in the same kind of a Pyrex test-tube and in the manner already described. 1 or 2 cc. of 10 per cent sodium hydroxide are added and the ammonia is aspirated into a test-tube graduated at 25 cc. and containing 2 cc. of 0.05 normal hydrochloric acid. The only precaution which experienced investigators are likely to overlook is that the rubber tubing used for connections needs to be rinsed with water before being used the first time, and, later also, if the tubing has been idle for any length of time. The talcum powder with which the inner and outer surface of rubber tubing is coated is probably the source of the trouble in the case of new rubber tubing. It is probably contaminated with ammonia.

Urea Determination by Means of Autoclave Decomposition.—To 5 cc. of blood filtrate in a 75 cc. test-tube is added 1 cc. of normal acid; the mouth of the test-tube is covered with tin-foil, and the

test-tube with contents is then heated in the autoclave at 150°C. for 10 minutes.

Allow the autoclave to cool to below 100°C. before opening. The ammonia is then distilled off exactly as in the first process described except that 2 cc. of 10 per cent sodium carbonate are substituted for the borax or it is removed by aeration in the usual manner. The autoclave process is of course only an adaptation of the process first recommended and then abandoned by Benedict for the determination of urea in urine. We are not prepared to say that in terms of per cent the results may not be as much too high in our blood filtrates as they were found to be in urine. An error of several per cent is, however, not at all important in the determination of the urea in blood. Whether one finds 15 instead of 14 mg. of urea nitrogen in human blood, or whether one obtains 3 instead of 2 mg. in 100 cc. of chicken blood is as yet of comparatively small consequence.

Determination of Creatinine and Creatine.

In this section we shall describe fairly obvious applications to our blood filtrate of Folin's colorimetric method for the determination of creatinine and creatine without thereby implying that the results so obtained are more accurate than the results which can be obtained by various other modifications which have been proposed during the past 2 or 3 years. The original methods as applied to blood were devised for the purpose of studying the absorption of creatinine and creatine and were adequate for that problem. Subsequent experience in many laboratories has shown that the method for the creatine gives results that are too high. The false step introduced in connection with the creatine determinations was undoubtedly the employment of picric acid as a protein precipitant, although at the time this seemed a peculiarly suitable process for securing the creatinine in concentrations then deemed necessary for reliable color comparisons. The process could perhaps be saved if it were worth while, for the cause of the high results is probably the formation of traces of hydrogen sulfide during the heating in the autoclave. The method is, however, now superfluous.

Determination of Preformed Creatinine.—Transfer 25 (or 50) cc. of a saturated solution of purified picric acid to a small, clean flask,

add 5 (or 10) cc. of 10 per cent sodium hydroxide, and mix. Transfer 10 cc. of blood filtrate to a small flask or to a test-tube, transfer 5 cc. of the standard creatinine solution described below to another flask, and dilute the standard to 20 cc. Then add 5 cc. of the freshly prepared alkaline picrate solution to the blood filtrate, and 10 cc. to the diluted creatinine solution. Let stand for 8 to 10 minutes and make the color comparison in the usual manner, never omitting first to ascertain that the two fields of the colorimeter are equal when both cups contain the standard creatinine picrate solution. The color comparison should be completed within 15 minutes from the time the alkaline picrate was added; it is therefore never advisable to work with more than three to five blood filtrates at a time.

When the amount of blood filtrate available for the creatinine determination is too small to permit repetition, it is of course advantageous or necessary to start with more than one standard. If a high creatinine should be encountered unexpectedly without several standards ready, the determination can be saved by diluting the unknown with an appropriate amount of the alkaline picrate solution—using for such dilution a picrate solution first diluted with two volumes of water—so as to preserve equality between the standard and the unknown in relation to the concentration of picric acid and sodium hydroxide.

One standard creatinine solution, suitable both for creatinine and for creatine determinations in blood, can be made as follows: Transfer to a liter flask 6 cc. of the standard creatinine solution used for urine analysis (which contains 6 mg. of creatinine); add 10 cc. of normal hydrochloric acid, dilute to the mark with water, and mix. Transfer to a bottle and add four or five drops of toluene or xylene. 5 cc. of this solution contain 0.03 mg. of creatinine, and this amount plus 15 cc. of water represents the standard needed for the vast majority of human bloods, for it covers the range of 1 to 2 mg. per 100 cc. In the case of unusual bloods representing retention of creatinine, take 10 cc. of the standard plus 10 cc. of water, which covers the range of 2 to 4 mg. of creatinine per 100 cc. of blood; or 15 cc. of the standard plus 5 cc. of water by which 4 to 6 mg. can be estimated. By taking the full 20 cc. volume from the standard solution at least 8 mg. can be estimated; but when working with such blood it is well to consider whether

it may not be more advantageous to substitute 5 cc. of blood filtrate plus 5 cc. of water for the usual 10 cc. of blood filtrate.

Calculation.—The reading of the standard in mm. (usually 20) multiplied by 1.5, 3, 4.5, or 6 (according to how much of the standard solution was taken), and divided by the reading of the unknown, in mm., gives the amount of creatinine, in mg. per 100 cc. of blood. In connection with this calculation it is to be noted that the standard is made up to twice the volume of the unknown, so that each 5 cc. of the standard creatinine solution, while containing 0.03 mg., corresponds to 0.015 mg. in the blood filtrate.

Determination of Creatine plus Creatinine.—Transfer 5 cc. of blood filtrate to a test-tube graduated at 25 cc. These test-tubes are also used for urea and for sugar determinations. Add 1 cc of normal hydrochloric acid. Cover the mouth of the test-tube with tin-foil and heat in the autoclave to 130°C. for 20 minutes or, as for the urea hydrolysis, to 155°C. for 10 minutes. Cool. Add 5 cc. of the alkaline picrate solution and let stand for 8 to 10 minutes, then dilute to 25 cc. The standard solution required is 20 cc. of creatinine solution in a 50 cc. volumetric flask. Add 2 cc. of normal acid and 10 cc. of the alkaline picrate solution and after 10 minutes standing dilute to 50 cc. The preparation of the standard must of course have been made first so that it is ready for use when the unknown is ready for the color comparison. The height of the standard, usually 20 mm., divided by the reading of the unknown and multiplied by 6 gives the "total creatinine" in mg. per 100 cc. blood.

In the case of uremic bloods containing large amounts of creatinine 1, 2, or 3 cc. of blood filtrate, plus water enough to make approximately 5 cc., are substitutes for 5 cc. of the undiluted filtrate.

The normal value for "total creatinine" given by this method is about 6 mg. per 100 cc. of blood.

Determination of Uric Acid.

The colorimetric method for the determination of uric acid in blood, like the colorimetric method for the determination of creatinine in urine, has furnished a tangible starting point for much important research. With the introduction by Benedict of po-

tassium cyanide (or, as we prefer, sodium cyanide) for dissolving the silver urate, the uric acid method was materially simplified, and a new impetus was given to a widespread use of the process in researches of various kinds. For about 3 years doubts have been strong in this laboratory as to whether the method is really as reliable as it was at first believed to be, and in this laboratory at least we decided not to make further applications of it in research until these doubts could be removed. Our misgivings have proved in part unfounded and in part correct. Our fear that relatively large traces of uric acid are carried down with the blood proteins, during the coagulation process, have proved substantially groundless. On the other hand, it is certainly true that the precipitation of the uric acid from the concentrated blood filtrates by means of magnesia mixture and silver lactate (*i.e.*, essentially

CORRECTION.

On page 100, Vol. XXXVIII, No. 1, May, 1919, line 19, for *20 cc. of creatinine solution*, read *10 cc. of creatinine solution*.

nated almost wholly by taking aliquot portions of the blood filtrate (instead of the whole plus wash water). As a control method we have found the following process useful.

Heat about 160 cc. of water to boiling in a previously weighed beaker (capacity 500 cc.). Add 2 cc. of normal acetic acid, and add with pipettes 40 cc. of blood. Heat with constant stirring until the mixture is again boiling and continue the boiling for 2 minutes. Transfer beaker and contents to the scales, and add water until the total weight of the contents amounts to 200 gm. Mix and filter immediately. Transfer 100 cc. of the water-clear filtrate to an evaporating dish, add 1 cc. of 25 per cent acetic acid,

it may not be more advantageous to substitute 5 cc. of blood filtrate plus 5 cc. of water for the usual 10 cc. of blood filtrate.

Calculation.—The reading of the standard in mm. (usually 20) multiplied by 1.5, 3, 4.5, or 6 (according to how much of the standard solution was taken), and divided by the reading of the unknown, in mm., gives the amount of creatinine, in mg. per 100 cc. of blood. In connection with this calculation it is to be noted that the standard is made up to twice the volume of the unknown, so that each 5 cc. of the standard creatinine solution, while containing 0.03 mg., corresponds to 0.015 mg. in the blood filtrate.

Determination of Creatine plus Creatinine.—Transfer 5 cc. of blood filtrate to a test-tube graduated at 25 cc. These test- [illegible] determinations. Add

In the case of [illegible]
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The normal value for "total creatinine" given by this method is about 6 mg. per 100 cc. of blood.

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Heat about 160 cc. of water to boiling in a previously weighed beaker (capacity 500 cc.). Add 2 cc. of normal acetic acid, and add with pipettes 40 cc. of blood. Heat with constant stirring until the mixture is again boiling and continue the boiling for 2 minutes. Transfer beaker and contents to the scales, and add water until the total weight of the contents amounts to 200 gm. Mix and filter immediately. Transfer 100 cc. of the water-clear filtrate to an evaporating dish, add 1 cc. of 25 per cent acetic acid,

and boil down as rapidly as possible to a volume of about 5 cc. Transfer the residue to a 15 cc. centrifuge tube, rinsing with 1 to 2 cc. of 0.1 per cent lithium carbonate solution. Cool. Add 2 cc. of Benedict's ammoniacal silver magnesia mixture, stir for 2 minutes, and centrifuge. Decant as completely as possible the supernatant liquid. To the residue in the tube add 2 cc. of 5 per cent sodium cyanide solution; stir, add 10 to 13 cc. of water, stir, and centrifuge again. Transfer the supernatant liquid to a 100 cc. volumetric flask, and make the color comparison in the usual manner.

As a control method the process outlined above is good, and if we were dependent on the method for regular use we should cut it down and introduce corrections for the solubility of silver urate.

Before describing the determination of uric acid in tungstic acid blood filtrates we wish to describe the preparation of a new standard solution of uric acid—a solution the keeping quality of which we now, after 18 months of constant use, consider much superior to any other as yet devised. The solvent is 10 per cent sodium sulfite, and the keeping quality of the solution depends on the fact that the sulfite keeps the solution free from dissolved oxygen. The solution is prepared as follows:

Make 1 to 3 liters of a 20 per cent solution of sodium sulfite, let stand over night, and filter. Dissolve 1 gm. of uric acid in 125 to 150 cc. of 0.4 per cent lithium carbonate solution and dilute to a volume of 500 cc. Transfer 50 cc., corresponding to 100 mg. of uric acid, to each of a series of volumetric liter flasks. Add 200 to 300 cc. of water, then 500 cc. of filtered 20 per cent sodium sulfite solution, and finally make up to volume, and mix well. Fill a series of 200 cc. bottles, and stopper very tightly with rubber stoppers. The solution in a bottle which is opened daily will keep for at least 3 to 4 months. Our records kept for one larger bottle so used show that no measurable loss of uric acid had occurred at the end of 6 months. In unopened bottles we expect the uric acid to keep for many years.

The surplus 20 per cent sulfite solution should be diluted to concentration of 10 per cent and should then be transferred to another series of small, tightly stoppered bottles. This sulfite is added to the unknown in order to offset the sulfite content of the standard.

Solutions Required for Uric Acid Determinations.

1. The standard uric acid sulfite solution already described (3 cc. used for each series of determinations).

2. A 10 per cent sodium sulfite solution, also described (2 cc. used for each determination).

3. A 5 per cent sodium cyanide solution, to be added from a burette (2.5 to 5 cc. used for each series of determinations).

4. A 10 per cent solution of sodium chloride in 0.1 normal hydrochloric acid (10 to 20 cc. used for each series of determinations).

5. The uric acid reagent prepared according to Folin and Denis. A still stronger reagent is obtained by heating the sodium tungstate (100 gm.) and the phosphoric acid (80 cc.) plus water (700 cc.) for 24 hours, instead of 2 hours; but the advantage gained, about 20 per cent, is not needed. Dilute the solution to 1 liter.

6. A solution of 5 per cent silver lactate in 5 per cent lactic acid (4 to 5 cc. needed for each determination).

In our new method for the determination of uric acid the latter is precipitated directly from the filtrate, without any previous concentration. 20 cc. of filtrate corresponding to 2 cc. of blood are used. In describing the process we shall have to introduce a slight variation from the way we actually do it. This variation is due to the fact that we use a larger centrifuge than most laboratories possess and by means of which we are able to use 30 cc. test-tubes for the precipitation. Using the small 15 cc. centrifuge tubes, it is necessary either to precipitate 10 cc. of filtrate in each of two tubes or to make the precipitation in two 10 cc. installments.

To 10 cc. of blood filtrate in each of two centrifuge tubes add 2 cc. of a 5 per cent solution of silver lactate in 5 per cent lactic acid, and stir with a very fine glass rod. Centrifuge; add a drop of silver lactate to the supernatant solution, which should be almost perfectly clear and should not become turbid when the last drop of silver solution is added. Remove the supernatant liquid by decantation as completely as possible. Add to each tube 1 cc. of a solution of 10 per cent sodium chloride in 0.1 normal hydrochloric acid and stir thoroughly with the glass rod. Then add 5 to 6 cc. of water, stir again, and centrifuge once more. By this chloride treatment the uric acid is set free from the precipitate. Transfer the two supernatant liquids by decantation to a 25 cc. volu-

metric flask. Add 1 cc. of a 10 per cent solution of sodium sulfite, 0.5 cc. of a 5 per cent solution of sodium cyanide, and 3 cc. of a 20 per cent solution of sodium carbonate. Prepare simultaneously two standard uric acid solutions as follows:

Transfer to one 50 cc. volumetric flask 1 cc. and to another 50 cc. flask 2 cc. of the standard uric acid sulfite solution described above. To the first flask add also 1 cc. of 10 per cent sodium sulfite solution. Then add to each flask 4 cc. of the acidified sodium chloride solution, 1 cc. of the sodium cyanide solution, and 6 cc. of the sodium carbonate solution. Dilute with water to about 45 cc. When the two standard solutions and the unknown have been prepared as described they are ready for the addition of the uric acid reagent of Folin and Denis. Add 0.5 cc. of this reagent to the unknown and 1 cc. to each of the standards, and mix. Let stand for 10 minutes, fill to the mark with water, mix, and make the color comparison.

Calculation.—In connection with the calculation it is to be noted (*a*) that the blood filtrate taken corresponds to 2 cc. of blood, (*b*) that the standard is diluted to twice the volume of the unknown, and (*c*) that the standard used contains 0.1 or 0.2 mg. of uric acid. The blood filtrate from blood containing 2.5 mg. of uric acid will be just equal in color to the weaker standard. 20 times 2.5 divided by the reading of the unknown gives, therefore, the uric acid content of the blood when the weaker standard is set at 20 mm.

The two standards recommended were adopted on the basis of the experience gained from the analysis of more than 150 different samples of human blood. About one-third of these bloods was from soldiers and most of the others were obtained from the State Wassermann Laboratory through the courtesy of Dr. Hinton. The bloods unfortunately do not cover the wider range occurring among hospital patients. A moderate number of blood samples have been obtained from the Massachusetts General Hospital, and these reveal that the uric acid may sink to as low as 1 mg. of uric acid per 100 cc. of blood. It seems hardly worth while to prepare a third and weaker standard regularly in order to provide for such low uric acid values. A standard corresponding to the color obtained from 1.25 mg. of uric acid per 100 cc. of blood can be prepared within a couple of minutes as follows:

Transfer 1 cc. of 10 per cent sulfite solution, 3 cc. of 20 per cent sodium carbonate, 2 cc. of the acidified sodium chloride, 0.5 cc. of the sodium cyanide solution, and 25 cc. of the weaker one of the two regular standard solutions already on hand. Dilute to 50 cc. and mix. Or, simply add 5 cc. of 20 per cent sodium carbonate to 25 cc. of the regular weaker standard, and dilute to 50 cc.

If a low uric acid value is expected, an alternate procedure is to dilute the unknown to a final volume of 10 cc. with corresponding reduction in the amount of the reagents used.

Special attention should perhaps be called to one small yet essential variation in the process for developing the blue uric acid

TABLE III.

Comparison of Old and New Methods For Determination of Blood Uric Acid.

Source.	Mg. per 100 cc. blood.		
	New method.	Old method.*	
		Without solubility correction.	With solubility correction.
Human	2.6	2.2	2.4
"	3.5	3.4	3.6
Chicken	2.8	2.7	2.9
"	3.4	3.3	3.5
"	2.5	2.3	2.4
"	3.8	3.5	3.7

* Slightly modified.

color, a variation made necessary by the use of sodium sulfite. The uric acid reagent must invariably be added after, and not before, the addition of the sodium carbonate, because in acid solution the sulfite will itself give a blue color with the phosphotungstic acid.

It may also be worth while to mention that the peculiar increase in blue color obtained by the use of cyanide is not obtained in the presence of sulfite. Opinions will doubtless differ as to whether this is an advantage or disadvantage. The amount of color obtained from 2 cc. of blood is rather weak, and if we could conveniently have retained the intensifying effects of the cyanide we probably should have done so, though the fainter solutions can be

read just as readily and accurately as the stronger ones obtained by means of the cyanide. The antifading effects of the cyanide are retained.

New Method for Determination of Sugar.

It was originally our intention to incorporate some adaptation of Benedict's picrate method for the determination of sugar to our tungstic acid blood filtrates. But a few exploratory experiments showed that an intense and stable color reaction can be obtained by the application of the phenol reagent of Folin and Denis to cuprous oxide. The color obtained from a given quantity of sugar is far more intense than that obtained by the alkaline picrate reaction; so that a small fraction of a mg. of dextrose (1 or 2 cc. of blood filtrate) is all that is required for a determination of the blood sugar. Some difficulties were encountered in trying to find the conditions under which the extent of reduction is strictly proportionate to the quantities of sugar used; but, by a systematic study of the various factors involved, these difficulties were overcome and a rapid and convenient process was obtained.

The copper solution used for reduction is a weakly alkaline copper tartrate solution. Qualitatively this solution is an extremely sensitive reagent for traces of sugar, yet is not affected by creatinine or uric acid in quantities corresponding to 50 mg. of each per 100 cc. of blood. We are therefore inclined to regard our method as more accurate than any method as yet proposed for the determination of sugar in blood.

The picrate methods,[7] whether we use Benedict's last modification or Myers' modification of Benedict's original method, in our hands give almost invariably results that are materially higher than the figures given by our new method. We are under the impression that the picrate methods are subject to sources of error similar to those encountered in Folin's original picrate method for blood creatine. The development of color in blood filtrates seems

[7] Lewis, R. C., and Benedict, S. R., A Method for the estimation of sugar in small quantities of blood, *J. Biol. Chem.*, 1915, xx, 61. Myers, V. C., and Bailey, C. V., The Lewis and Benedict method for the estimation of blood sugar, with some observations obtained in disease, *ibid.*, 1916, xxiv, 147. Benedict, S. R., A modification of the Lewis-Benedict method for the determination of sugar in the blood, *ibid.*, 1918, xxxiv, 203.

not to proceed at the same rate of speed as the color derived from a corresponding amount of dextrose. If the heating is interrupted at the end of 2 to 3 minutes the value obtained for the blood sugar will be nearly 50 per cent higher than when the heating is continued for 10 minutes or more. Such quantitative variations are not encountered in our process when equal amounts of dextrose in the form of pure sugar and of blood filtrate are heated, except that the reduced copper is, of course, more extensively precipitated and visible in the pure sugar solution. It need scarcely be stated that added sugar is quantitatively recovered by our method.

Solutions Needed for Determination of Sugar in Blood.

1. *Standard Sugar Solution.*—Dissolve 1 gm. of pure anhydrous dextrose in water and dilute to a volume of 100 cc. Mix, add a few drops of xylene or toluene, and bottle. If pure dextrose is not available, a standard solution of invert sugar made from cane sugar is equally useful. Transfer exactly 1 gm. of cane sugar to a 100 cc. volumetric flask; add 20 cc. of normal hydrochloric acid and let the mixture stand over night at room temperature (or rotate the flask and contents continuously for 10 minutes in a water bath kept at 70°C.). Add 1.68 gm. of sodium bicarbonate and about 0.2 gm. of sodium acetate, to neutralize the hydrochloric acid. Shake a few minutes to remove most of the carbonic acid and fill to the 100 cc. mark with water. Then add 5 cc. more of water (1 gm. of cane sugar yields 1.05 gm. of invert sugar) and mix. Transfer to a bottle; add a few drops of xylene or toluene, shake well, and stopper tightly. The stock solution made in either way keeps indefinitely. Dilute 5 cc. to 500 cc., giving a solution 10 cc. of which contain 1 mg. of dextrose or invert sugar. Add some xylene. Use 2 cc. for each determination.

2. *Alkaline Copper Solution.*—Dissolve 40 gm. of anhydrous sodium carbonate in about 400 cc. of water and transfer to a liter flask. Add 7.5 gm. of tartaric acid and when the latter has dissolved add 4.5 gm. of crystallized copper sulfate; mix, and make up to a volume of 1 liter. If the carbonate used is impure, a sediment may be formed in the course of a week or so. If this happens, decant the clear solution into another bottle.

3. *Phosphotungstic-phosphomolybdic Acid.*—Transfer to a large flask 25 gm. of molybdenum trioxide (MoO_3) or 34 gm. of ammonium molybdate $(NH_4)_2(MoO_4)$; add 140 cc. of 10 per cent sodium hydroxide and about 150 cc. of water. Boil for 20 minutes to drive off the ammonia (molybdic acid sometimes contains large amounts of ammonia as impurity). Add to the solution 100 gm. of sodium tungstate, 50 cc. of 85 per cent phosphoric acid, and 100 cc. of concentrated hydrochloric acid. Dilute to a volume of 700 to 800 cc.; close the mouth of the flask with a funnel and watch-glass. Boil gently for not less than 4 hours, adding hot water from time to time to replace that lost during the boiling. Cool and dilute to 1 liter. This solution is identical with the phenol reagent of Folin and Denis. For use in connection with the determination of blood sugar dilute 1 volume (100 cc.) of the reagent with one-half volume (50 cc.) of water and one-half volume (50 cc.) of concentrated hydrochloric acid.

4. *Saturated Sodium Carbonate Solution.*

The determination of blood sugar is carried out as follows: Heat a beaker of water to vigorous boiling. Transfer 2 cc. of the tungstic acid blood filtrate to a test-tube (20 m. × 200 mm.) graduated at 25 cc. The graduated test-tubes used as receivers when distilling off the ammonia in urea determinations (p. 95) are suitable for this work. Transfer 2 cc. of the dilute standard sugar solution to another similar test-tube. Add to each tube 2 cc. of the alkaline copper tartrate solution. Heat in the boiling water for 6 minutes. Remove the test-tube and add at once (without cooling), preferably from a graduated pipette, 1 cc. of the strongly acidified and diluted phenol reagent. This should be done as nearly simultaneously as possible; it is not advisable to use one standard for a set of more than four determinations. The purpose of the added hydrochloric acid in the reagent is to dissolve the cuprous oxide. Mix, cool, and add 5 cc. of saturated sodium carbonate solution. An intense blue color is gradually developed which will remain unaltered for several days. Dilute the contents of both test-tubes to the 25 cc. mark, and after at least 5 minutes make the color comparison in the usual manner.

The depth of the standard (in mm.) multiplied by 100 and divided by the reading of the unknown gives the sugar content, in mg., per 100 cc. of blood.

TABLE IV.

Sample Analyses of Protein-Free Blood Filtrates Obtained by Means of Tungstic Acid.

No.	Mg. per 100 cc. blood.					
	Total N.	Urea N.	Uric acid.	Preformed creatinine.	Total creatinine.	Sugar.
1	26	10	1.3	1.5	6.0	89
2	26	13	1.0	1.4	5.3	100
3	28	12	1.1	1.2	6.7	98
4	28	12	2.2	2.0	5.7	83
5	29	13	3.3	1.5	6.0	86
6	29	11	2.6	1.4	5.2	95
7	29	13	1.6	1.4	6.0	85
8	30	13	2.4	1.6	5.5	82
9	30	14	4.1	1.7	5.3	82
10	32	15	2.8	1.6	5.4	91
11	32	15	3.4	1.4	5.3	97
12	32	13	2.4	1.7	6.0	104
13	33	17	2.0	1.3	4.8	83
14	33	16	2.5	1.6	5.7	105
15	33	15	1.1	1.6	5.5	95
16	34	16	0.8	1.3	6.1	119
17	34	16	2.6	1.5	5.9	106
18	35	17	2.1	1.6	6.0	89
19	35	17	2.0	1.4	5.5	77
20	35	18	2.0	1.7	5.7	86
21	35	18	2.9	1.6	5.8	95
22	35	17	3.2	1.4	5.5	94
23	35	18	2.5	1.5	6.0	89
24	35	19	2.2	1.5	5.3	91
25	35	22	3.5	1.4	5.7	87
26	35	17	2.3	1.7	6.7	83
27	35	18	1.6	1.3	6.5	104
28	36	17	2.8	1.5	5.2	100
29	37	18	2.1	1.5	5.5	94
30	38	18	2.2	1.7	5.4	95
31	39	18	2.6	1.8	6.7	103
32	39	18	2.9	1.5	6.0	87
33	40	18	2.0	1.6	6.0	98
34	40	20	2.6	1.7	5.6	95
35	41	19	4.8	1.5	5.9	93
36	41	19	4.2	2.5	6.6	109
37	43	19	2.2	1.7	6.3	78
38	139	106	5.4	12.5	19.4	99
39	147	115	8.9	11.0	20.5	170
40	275	237	14.3	13.6	27.2	157

The copper solution is adjusted to give proportionate reductions with 0.12 to 0.4 mg. of dextrose. This covers the range of hypoglycemic and hyperglycemic bloods. But in extreme cases it is better to use 3 or 1 cc. of the filtrate, instead of 2 cc., adding water to the standard or to the unknown so as to equalize the concentration of the alkaline copper.

NOTE ON THE DETERMINATION OF UREA IN URINE BY DIRECT NESSLERIZATION.

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Merck's blood charcoal was a necessary reagent in determination of urea in urine by the direct Nesslerization process of Folin and Denis. By using a urease preparation sufficiently free from nitrogenous materials the urea nitrogen can be Nesslerized without any charcoal treatment. The process is as follows:

Wash about 3 gm. of permutit in a flask once with 2 per cent acetic acid, then twice with water; add 5 gm. of fine jack bean meal and 100 cc. of 30 per cent alcohol. Shake gently but continuously for 10 to 15 minutes and filter. The filtrate contains practically the whole of the urease and extremely little of other materials. Add 1 cc. of this urease solution to 1 cc. of diluted urine (dilution usually 1: 10) in a test-tube and digest in a beaker of warm water (40–55°C.) for 5 minutes or at room temperature for 15 minutes. It is preferable, but not necessary, to add a drop of one of the phosphate solutions described in the preceding paper[1] to the mixed contents in the test-tube at the beginning of the digestion. The buffer mixture is particularly desirable if the digestion is to be made at room temperature. At the end of the digestion period transfer the contents of the test-tube to a 200 cc. volumetric flask, diluting to a volume of about 150 cc. Add 1 mg. of N in the form of ammonium sulfate to another 200 cc. flask; to this standard add 1 cc. of the urease solution and dilute to about 150 cc. Then add 20 cc. of the Nessler solution prepared according to the directions given in the preceding paper (p. 89). Dilute to volume and make the color comparison. The ammonia nitrogen is, of course, included in the figure obtained.

A few explanatory remarks may be added. Many kinds of biological nitrogenous materials, particularly amino-acids, pep-

[1] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

tones, and albumins, prevent the development of the color reaction given by ammonia and Nessler's reagent. This was first discovered in attempts to determine by direct Nesslerization the ammonia formed in pancreatic digestion mixtures. If very little such nitrogenous material is present, the result obtained is deceptive for then the color is merely diminished and the error will not be detected. The careful observer will find, however, that in such cases the color obtained tends to be visibly more greenish and less distinctly red than the standard. Because of the serious interference caused by albuminous materials it may be thought that the procedure described above is not applicable to albuminous urines, but by a series of determinations we have convinced ourselves that even urines very rich in albumin have in fact so little in comparison with the amount of urea present that correct results are invariably obtained by direct Nesslerization.

Because of the extremely low nitrogen content of our urease preparation, it is not really essential that the urease should also be added to the standard ammonia solution, but we have thought it best to recommend that it be added simply as a precaution against the possible occurrence of less good urease preparations. In recommending the addition of the urease to the standard as well as to the urine we have also had in mind the probability that many will omit the use of the permutit when making the alcoholic urease extracts and will then have variable small traces of ammonia in their extracts.

The reason why the urease decomposition is better made in test-tubes rather than in volumetric flasks is to avoid failure due to the use of flasks which have been used for Nesslerization purposes. Such flasks may look perfectly clean, but, unless they are rinsed with nitric acid, they will contain enough mercury compounds to destroy entirely the urease and scarcely a trace of ammonia is obtained.

The method described above was worked out about 2 years ago and has been used with our medical students for two seasons. The publication has been delayed because it was our intention to incorporate it in a larger research. After consultation with Dr. W. S. McEllory who has independently devised a method very similar to ours, it has seemed better to publish the method without further delay.

A BIOLOGICAL ANALYSIS OF PELLAGRA-PRODUCING DIETS.

VI. OBSERVATIONS ON THE FAULTS OF CERTAIN DIETS COMPARABLE TO THOSE EMPLOYED BY MAN IN PELLAGROUS DISTRICTS.

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PLATE 1.

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In making an analysis of the relation of the diet to pellagra, the results of which have been presented in the present series of papers, we began by showing the nature of the dietary faults of a mixture of two seeds, one a cereal (maize) and the other a legume seed (navy bean). It was shown that with this mixture of two natural foodstuffs, which are both recognized as wholesome foods, the same kinds of deficiencies exist as we had previously determined for each of the more important seeds employed as foodstuffs when fed as the sole source of nutrients; *viz.*, a decided shortage of the mineral elements, calcium, sodium, and chlorine; a relative shortage of the dietary factor fat-soluble A; and a protein content of rather low biological value as compared with such foods as milk, meats, and eggs. The detailed evidence in support of these deductions was presented in the first paper of the series (1). The first limiting factor in determining the nutritive value of a mixture of maize and navy beans is the mineral content, and the remaining two factors which are unsatisfactory are each below the optimum to about the same degree. All three of these factors must be supplemented before satisfactory nutrition can be secured.

We had previously made the observation that fairly good nutrition, as shown by capacity to grow to the full adult size and produce and rear young, could be secured with monotonous diets derived from mixtures of the leaf of the plant with the seed (2), whereas with mixtures of seeds only little or no growth could be

secured in young animals (3), and adult animals fail to maintain their vitality when restricted to monotonous diets of this character (4). In the fourth paper of the series the dietary properties of mixtures of seeds derived from three to five sources were made clear. It was demonstrated that there is almost no supplementary action between the ordinary seeds which are employed as human foods with respect to the mutual improvement of their inorganic content, and that they do not supplement each other with respect to fat-soluble A (3).

That the proteins of the seeds which are of importance in nutrition are of low biological value for the support of growth was made clear by more elaborate experiments in the third paper of the series (5). In the second paper (6) it was demonstrated that physiological well being is not maintained when animals are limited to an intake of any one or more dietary factors, allowing but a small margin over the actual daily requirements for the repair of the endogenous waste. In a recent communication it has likewise been made clear that the supplementary relationship between the proteins of the seeds generally is much lower than one might logically expect, so that the protein mixture derived from a diet containing seeds from a number of different plants is in general of low value for transformation into body proteins. There have been observed however, a few mixtures of two seeds in certain proportions, in which the quality of the proteins is nearly if not quite the equivalent of milk proteins in value (7).

The solution of the problems discussed in the foregoing papers forms a basis for deductions of far reaching importance in the relation of the diet to pellagra. Our data all support the view that with the exception of a single dietary complex (fat-soluble A) each whole seed which is of importance in the nutrition of man in America contains sufficient of any one or more chemically unidentified dietary factors to supply the needs of the growing young rat. According to the working hypothesis adopted by McCollum and Davis (8), and adhered to as the simplest one by means of which our experimental data with the rat could be interpreted, there are but two such complexes; *viz.*, the substance which is protective against xerophthalmia (fat-soluble A), and that which is protective against beri-beri (water-soluble B).

Our experimental studies have now progressed so far as to enable us to assert with confidence that a satisfactory diet cannot be secured from mixtures containing any number of seeds or products derived from the milling of seeds together with tubers, edible roots, and meats (Chart 4, Lot 2147). The vegetable foods which may be classed as seeds, tubers, and roots are all functionally storage organs, and their content of active protoplasm is relatively small in comparison with their bulk because of the large amount of reserve food material laid down in them. They may be sharply contrasted with the leaf of the plant, which except in special cases is not a repository for reserve proteins, carbohydrates, and fats, but represents, aside from its skeletal tissues, functionally active protoplasm (9). The leaf has very different dietary properties from those possessed by the tissues which are modified as storage organs, and in many instances at least represents complete foods for those types of animals whose digestive tracts are so capacious as to permit them to eat a sufficient amount of bulky material. As stated above, we have been able to prepare fairly satisfactory diets for an omnivorous animal, the rat, from these two types of vegetable foods together, *i.e.*, leaves and seeds, but never from the group of vegetable foods which are functionally storage organs (10).

From this experience we have been led to differentiate sharply between two classes of foods which are usually collectively designated as vegetables. Leaves are constituted so as to correct the dietary deficiencies of the storage tissues, whereas the seeds, tubers, and roots fail to supplement mutually each other's deficiencies with respect to either the inorganic moiety or the fat-soluble A. They do in some degree mutually enhance the quality of each other's proteins, but to a lesser degree than we had supposed before the completion of a large amount of experimental work directed toward the quantitative comparison of the protein mixtures derived from pairs of seeds in considerable number.

Mixtures of seeds, or of seeds, tubers, and roots, will in all cases require supplementing with respect to calcium, sodium, and chlorine among the inorganic elements, and fat-soluble A (compare Charts 3 and 4 with Chart 5). In most such mixtures the quality of the proteins will likewise be sufficiently poor to require improvement before the optimum well being can be secured.

We are now in possession of a considerable amount of knowledge concerning the distribution of the dietary factor, fat-soluble A, in animal tissues. The body fats of the ruminants probably always will be found to be richer in this substance than the body fats of the omnivora because of the greater intake of it in the food (11). Muscle tissue has been found to be very poor in fat-soluble A (4, 12) but the fats from the glandular organs, *i.e.*, intracellular fats, are a good source of it. It follows, therefore, that muscle tissue such as round steak should not supplement mixtures of vegetable foods which belong to the storage organ group with respect to fat-soluble A, and in our experience this proves to be the case. The inorganic content of muscle tissue resembles in a general way that of the storage organs of plants except in its very low content of magnesium. It is too poor in calcium, and to a lesser degree in sodium and chlorine, to support the optimum well being in an animal. Muscle tissue fails to supplement the seeds, tubers, and roots on the inorganic side (Chart 3). The protein content of muscle tissue is high and the proteins are probably of high biological value, and, except as respects palatability, it is only in improving the quality of the protein content of the ration that the addition of meats of this class enhances the value of a mixture of products derived from the storage organ group of plant products.

These considerations indicate the basis for our distinction between two groups of foodstuffs. One of these, which includes milk, eggs, and the leafy vegetables, we have designated as "protective foods," in order to call attention to their special importance in the diet (10). They are protective in that they are so constituted with respect to their inorganic content, content of fat-soluble A, and the quality of their proteins that they correct in great measure when used in sufficient amounts the faults of the remainder of the food mixture irrespective of the extent to which it is derived from either seed, tuber, or root products. We have been able to plan satisfactory diets of naturally occurring foods only by the inclusion of one or more of these protective foods. The other group of natural foodstuffs includes all seeds and seed products, such as the cereal grains and their milling products (wheat flour, corn-meal, polished rice, etc.), the legume seeds, tubers, edible roots, nuts, fruits, and such cuts of meats as come from muscle tissue.

In all cases where we have attempted to correct the dietary deficiencies of a seed mixture by the addition of leaf only we have not secured results so good as with milk, especially with such amounts of leaf as would be acceptable in the human diet (compare Charts 5 and 8). The leafy foods are eaten by Europeans and Americans only in a very water rich condition, and it is difficult to secure the consumption of enough to correct the deficiencies in the remainder of the diet. With animals, when we have fed dry powdered mixtures containing as much as 25 to 40 per cent of the diet derived from leaf and the remainder from plant products of the storage organ class, the nutrition has been very good in some instances, but not all combinations will be equally valuable (4). Eggs are decidedly poorer in calcium than are the leaves or milk, when only the part exclusive of the shell is considered. The shell serves as a source of lime to the developing chick. Eggs do not, therefore, supplement food mixtures derived from storage tissues with respect to calcium to the degree that milk and leafy vegetables do.

Even in such types of diet as contain one or more of the protective foods in fairly liberal amounts, it is certain that for such rapidly growing species of animals as the hog and rat the inorganic content is not entirely satisfactory, although it may be good enough to enable the animal to perform all the functions of growth and reproduction in a way which, in the absence of definite knowledge of what the species is capable of, we should regard as normal. We have been accustomed to regard as normal an achievement in vigor and well being both in man and animals which falls far short of that seen in exceptional cases. The most important inorganic deficiency in seed, tuber, and meat mixtures is calcium, and this is so pronounced that we are of the opinion that even in those human dietaries in which such calcium-rich food as milk are used in fair liberality, the intake of calcium may be still below the optimum, and that a direct addition of this element in the form of the carbonate or lactate might be of distinct benefit in human nutrition except perhaps in those regions where the water is unusually rich in calcium salts. Since civilized man usually adds sodium chloride to his foods to suit the taste, the shortage of sodium and chlorine in the diet of man presents no problem. An addition of calcium could be most conveniently made to our

foods through the use of a mixture of equal parts of common salt and of calcium carbonate in the kitchen and on the table.

A question which has never been answered to the satisfaction of physiologists is: How much protein should the diet contain in order to maintain physiological well being? At about the time when the question was being most discussed, the chemistry of the proteins was developed to a point which made it clear that there were great differences in the biological values of the proteins from different sources, depending on their yields of certain amino-acids. This makes futile any attempt to establish a particular intake of protein which may represent the minimum, optimum, or maximum amount consistent with maintenance of "normal" vitality and health. The quality of the proteins must be known before anything can be said about the amount of protein necessary. From biological tests we now know that the proteins of the pea or navy bean are worth only about half as much for growth in the rat as are equal amounts of proteins from one of the cereal grains, and that the latter have about half the value for the conversion into body proteins which can be shown for the proteins of milk. The relative values of the proteins from different sources, as well as the absolute values of certain of them, are just now becoming appreciated.

There are two opposing views regarding the amount of protein which will produce the best results. Those who advocate the low protein diet point to the "specific dynamic action" of protein, through which it stimulates metabolism. They believe that a high consumption of protein furnishes pabulum for the development of an excessive growth of putrefactive bacteria, with the result that toxic or irritating products of the degradation of certain amino-acids are absorbed in amounts sufficient to cause damage to the tissues (13). It has been recommended that man should, in adult life, take only such an amount of protein as will cover the endogenous loss due to tissue metabolism, together with a not well defined "margin of safety" (13). The opponents of this view regard a liberal protein allowance as essential to vigor and aggressiveness, and point to the use of liberal amounts of meat by the peoples who have been characterized by greatest achievement (14). *Among all the progressive peoples of the world the food supply is derived to a greater or less extent from dairy products,*

and this portion rather than the meat eaten we have come to regard as of peculiar importance in improving the quality of the diet. In order to test this question we conducted a series of experiments, employing rats which were about 9 months old, or about one-fourth through the normal span of life for this species, and were in excellent nutritive condition (5). They were fed diets which were fairly satisfactory in all respects except that the protein content was not far from the actual amount required for the maintenance of body weight for a few weeks. We observed unmistakable signs that the vitality of the animals was rapidly lowered on such a dietary régime. This was shown especially by the rapid aging and short span of life. Even though the initial body weight was approximately maintained for a period of 3 months or more, distinct signs of aging were always apparent within 5 to 10 months. 3 months in the life of a rat correspond to about 8.4 per cent of the average span of life. It can be readily appreciated that if harmful effects in corresponding degree follow the adherence by man to such low protein diets they would not become apparent within the time covered by any experiment yet conducted upon a diet squad, few of which have been restricted to any experimental diet beyond 6 months. A reputed satisfactory outcome of such experiments cannot be accepted as evidence that men on diets which furnish but a small margin of protein over the actual maintenance requirements are so nourished as best to promote health. Aging at two to four times the rate observed in the most satisfactorily nourished would escape observation in any experiment on man with which we are familiar.

The results of experiments with grown men restricted to experimental diets for a few weeks or months do not form a safe basis for drawing conclusions as to the quality of the foods employed. Certain conclusions may be warranted from general observations on children living on faulty diets, and important deductions may safely be drawn from the experiences of large groups of people living upon more or less restricted lists of foodstuffs. Beyond this we must be guided in human nutrition by the results of animal experimentation, in which the conditions can be made sufficiently rigid to bring into stronger contrast the faults of certain types of diets as contrasted with others. It is certain that the injurious effects of certain dietary practices are very real and yet not

promptly apparent. The debilitating effects of faulty diets may vary in their severity from such as will produce polyneuritis or xerophthalmia or scurvy within a few weeks, at one extreme, to such as will cause nervousness and restlessness in varying degree, susceptibility to disease, and the acquisition of all those characters such as roughness of the skin, thinness and coarseness of hair, and attenuation of form which accompany the process of aging at a distinctly greater rate than would be the case were the diet of a highly satisfactory character.

We have much evidence that in case there is a close approximation of the actual physiological minimum for any factor during growth, such as one or more of the essential inorganic elements or one of the unidentified dietary essentials, lack of ability to meet the more strenuous demands of reproduction and the suckling of young will be observed, and the tendency will be great for the individual to be carried off suddenly either by disease, or, as frequently happens, by causes which are not readily determinable (compare Charts 8 and 9).

All our experience with diets of low protein content have indicated that animals do not remain in a state of optimum well being even when the content of protein is sufficiently high to maintain in certain individuals the initial body weight over as much as 10 per cent of the normal span of life. We believe that health and vigor are promoted by a liberal intake of protein of good quality better than by any diet in which there is a tendency towards parcimony with respect to this dietary factor. It should not be lost sight of, however, that there are other factors in nutrition which are of equal importance with protein, and that if the optimum well being is to be attained the diet must be rightly constituted with respect to all its parts. In addition to this the prompt elimination of the fecal residues is essential and is a great relief to the tissues of the entire body.

With an understanding such as we now have of the nature of the faults of diets of different types, and an appreciation of the fundamental importance of deriving the constituents of the diet from the right sources, this being of much greater importance than composition as revealed by chemical analysis, one is in a position to interpret the relation of pellagra to diet.

Goldberger (15) has emphasized the fact that the diet of those living in districts in which pellagra is common is lacking in sufficient amounts of certain foodstuffs, especially milk, eggs, meats, and the legume seeds. In many instances bolted wheat flour, degerminated corn-meal, polished rice, sugar, syrups or molasses, sweet potatoes, and meat, principally pork, form almost the entire list of foods eaten by families during the winter season, at the end of which new attacks of pellagra are regularly seen. From what has been said it will be evident that the diet of the pellagrous is deficient in four respects, and that the nature of these is well understood. They are the deficiencies of the plant products which belong to the storage organ group, but more pronounced because of the prominent place which milling products, which represent the endosperm of the seed, find in such diets. Products such as bolted flour, degerminated corn-meal, and polished rice are decidedly poorer in inorganic elements than are the seeds from which they are derived; their proteins appear to be of poorer quality than are those of the cell-rich structures near the periphery, or of the germ, and they are almost devoid of fat-soluble A and very poor in water-soluble B. Whereas diets derived from whole seeds, tubers, and edible roots contain sufficient phosphorus to meet the requirements of the most rapidly growing species of animal, such as the rat, and the limiting inorganic elements are calcium, sodium, and chlorine, it may be that in diets in which the degerminated and decorticated cereal products are employed in liberal amounts, and where in addition starch, sugar, and molasses are regularly used freely, phosphorus or iron or both may likewise become important deficiencies (16).

Goldberger attempted to solve the problem of whether pellagra is due to lack of something essential in the typical "pellagrous" diet by a direct experiment on man (15). He restricted men to a diet prepared from bolted wheat flour, degerminated corn-meal, polished rice, starch, sugar, syrup, pork fat, sweet potatoes, cabbage, collards, turnip greens, and coffee, and at the end of $5\frac{1}{2}$ months five of the eleven men who took this diet were diagnosed as exhibiting incipient signs of pellagra. That the disease was actually produced has been emphatically denied by McNeal (17).

In another experiment Goldberger and fifteen of his associates made heroic attempts to infect themselves with material from the

lesions of pellagra, and with excreta from pellagrins, but without success. The experimenters were, however, taking a diet of good quality while these attempts were being made (18).

Still more convincing evidence that the diet is at least an important predisposing factor in the etiology of pellagra is furnished by the experience of Goldberger in improving the diets in institutions in which the disease was common. These diets were observed to consist largely of degerminated seed products, tubers or roots, and fat pork, together with minimal amounts of leafy vegetables, fruits, eggs, meats, and milk, and the legume seeds. On modifying the diets of orphanges and of an insane asylum by the addition of lean meat, milk, eggs, and peas or beans, the condition with respect to pellagra steadily improved, and the disease promptly disappeared. New cases were admitted from without and the sick were mingled with the well, but after the improvement of the diet no new cases developed (15).

Those who have had extensive experience with pellagra are in agreement in the matter of the fundamental importance of dietary treatment together with any other method of management of pellagrins, and the assertion has been made by Roussel that without dietary measures all remedies fail (19). The results obtained by Goldberger point clearly to the belief that the disease develops because of some one or more faults in the diet. They afford no basis, however, for judging as to the nature of these faults, whether they are in the nature of a lack of a sufficient amount of one or more chemically unidentified dietary essentials of a specific character, as is known to be the case with beri-beri and the xerophthalmia of dietary origin, or whether pellagra may be the result of taking a diet faulty in respect to the quality or quantity of protein, relative shortage of one or more of the essential inorganic elements, or of the recognized unidentified dietary essentials as contributing factors.

In his earlier papers Goldberger expressed the view that: "On the whole, however, the trend of available evidence strongly suggests that pellagra will prove to be a 'deficiency' disease very closely related to beri-beri" (15). Chittenden and Underhill (20) reported the production in dogs of a condition suggestive of pellagra in man by restricting the animals for periods of from 2 to 8 months to a diet of crackers, peas, and cottonseed oil. They

formulated the conclusion that: "From the facts enumerated the conclusion seems tenable that the abnormal state may be referred to a deficiency of some essential dietary constituent or constituents, presumably belonging to the group of hitherto unrecognized but essential components of an adequate diet."

In the fifth paper of this series we reported the results of a study of the nature of the dietary faults of a mixture of bolted wheat flour, peas, and cottonseed oil, and found that it was an incomplete food, but that it was rendered complete for the support of normal growth in the young rat by the addition of purified protein, certain inorganic salts (NaCl and $CaCO_3$) and fat-soluble A (in butter fat). It is of course not satisfactorily established that the condition produced in dogs by the diet of Chittenden and Underhill was actually the counterpart of pellagra in man, strikingly similar as the results appear. McCollum, Simmonds, and Parsons (21) hold the view that if the condition produced in the dogs of these investigators is actually to be regarded as experimental pellagra, it cannot be regarded as caused by the lack of an unidentified dietary essential, since the only one of these necessary for completing the diet (for the rat) is that contained in butter fat, and the latter substance is not curative for any condition resembling pellagra, but for a specific eye disease, xerophthalmia (6).

In his most recent studies Goldberger and his associates examined the diets of pellagrous and non-pellagrous families in villages in South Carolina, and found that the diet of the non-pellagrous contained more milk, fresh meats, eggs, butter, and cheese than did the diets of pellagrous families, and that the calorific value of the diets of the former households was somewhat higher than of the latter (22). Animal proteins were eaten more liberally and cereal proteins were eaten less abundantly by the non-pellagrous than by the pellagrous households. The pellagrous households had a distinctly smaller supply of fat-soluble A, and a somewhat smaller supply of water-soluble B than did the non-pellagrous, and the inorganic content of the diets of the latter were of less satisfactory character than those of the former households. *We do not regard a moderate shortage of one or another of the chemically unidentified dietary factors as of greater gravity than faulty character in any other dietary factor.* Our studies of the several foodstuffs lead us to agree with Goldberger's interpretation of the quality of the diets of pellagrous and non-pellagrous households in all respects.

From the observations which we have made concerning the chemical factors which the diet must contain in order to be adequate for the support of growth in the young, or the maintenance of physiological well being in the adult, together with the results of our studies of the qualities of each of the more important kinds of natural foodstuffs, we are not able to account for the etiology of pellagra on the assumption that it is a disease which is due to the lack of a specific substance or substances of unknown chemical nature, as are without question beri-beri and xerophthalmia (6). This follows from the fact that, with the exceptions of certain manufactured food products which are derived from the endosperm of the decorticated grains, *any natural foodstuffs of the class of seeds, tubers, edible roots, or leafy parts of the plant, are so constituted that they can be supplemented by means of three kinds of purified food additions of known nature; viz., protein, certain salts, and fat-soluble A, so as to be complete for the nutrition of the young rat throughout the growing period.* This has been demonstrated to be true not only for each of the ordinary human foods but likewise for such mixtures as form the monotonous diets of the pellagrous (see Charts 1, 2, 3, and 4).

It is necessary, therefore, that we choose between two alternatives in arriving at an opinion concerning the etiology of pellagra. We have the assurances of Goldberger and his associates (15) that a diet such as that described in Charts 1 to 3, and having the qualities described in the preceding paragraph, has produced incipient pellagra experimentally in man, but this claim has been disputed by other competent observers (17). In our experimental work with the diet of peas, crackers (wheat flour and fat), and cottonseed oil, which in the experience of Chittenden and Underhill produced in dogs a condition resembling pellagra in man, produced in rats only general malnutrition, without the skin changes, diarrhea, or pathological changes in the mucosa of the alimentary tract. Are we to accept the view that pellagra is actually produced by a deficiency of something necessary to the normal nutrition of man but not necessary for the rat? The possibility that the dogs of Chittenden and Underhill were infected is not excluded, and an infectious agent may well have established itself in animals restricted to a diet so faulty as one derived from crackers (wheat flour and fat), peas, and cottonseed oil. Goldberger seems to have

safeguarded his experimental men against infection, and it is unfortunate that a sufficient number of undisputed authorities were not called into consultation to forestall the possibility of a question arising concerning the accuracy of the diagnosis of pellagra, such as McNeal has raised (17).

We are left in the situation which has arisen in the discussion of the etiology of scurvy. It has been clearly shown that there is no difficulty in repeating the experimental work which demonstrated that a guinea pig will develop severe scurvy (or some syndrome resembling it) on a number of diets on which the rat will thrive during the growing period. Does this mean that the guinea pig requires one or more chemical complexes for its nutrition that are dispensable to the rat? There is no doubt that the guinea pig normally takes a diet rich in succulent vegetables, and which produces bulky, easily eliminable feces. The rat and swine, as well as man, thrive on certain diets which leave little indigestible residue. Such special requirements in the guinea pig make it next to impossible to compare this species with man or the rat in similar dietary studies. The experimental data obtained with the guinea pig must be used with caution in reasoning concerning the etiology of human scurvy.

In our attempts to produce in animals a condition analogous to pellagra in man we have not been successful, but have observed only a generalized poor condition instead. The evidence is practically nil that Chittenden and Underhill's dogs suffered all the pathological changes which they record solely as the result of chemical faults in the diet. Our experiments with their diet shows it to be incapable of maintaining satisfactory nutrition because of faults in the dietary factors. The possibility of an infection in their animals is not excluded, and is indeed rendered probable if we grant that lowered vitality predisposes to infection. These reasons together with the lack of positive proof that the men restricted in Goldberger's experiments to a diet similar to that described in Chart 1 were actually developing pellagra, warrant, we believe, our accepting as probably correct the conclusions of the Thompson-McFadden Commission (23) and of Jobling and Peterson (24) that pellagra is caused by an infectious agent, and that unless it has been introduced into a district there may develop such a condition of lowered vitality from faulty diet or other de-

bilitating influence as would predispose one to an attack, without the appearance of the disease. The debilitating effects on animals of diets derived from cereals, tubers, roots, and any food products formed from the milling of grains together with legume seeds and meats, are so striking that we believe similar diets would produce in man a susceptibility to infectious diseases such as tuberculosis or pellagra. We have come to hold the view, as the result of our studies of diets of the type common in pellagrous households, that the predisposing influence for both is in general the same, and the character of the unsanitary conditions surrounding the individual may determine which of these two diseases he will develop.

From the studies which we have described in this series of papers we are enabled to point out definitely the relative values of several foods as correctives in the diet of the pellagrous. Hitherto the legume seeds and lean meat were classed with milk and eggs in this respect, and nothing was said about the unique qualities of the leafy vegetables as supplements for food mixtures derived from plant products of the storage organ group. It is clear that the most important food to be recommended for consumption in pellagrous districts is milk, because of its cheapness as compared with the same protective value in foods from other sources and its threefold corrective character as contrasted with meat which enhances the type of diet found in the pellagrous household only with respect to the protein factor, and eggs which are not so good as milk because of their lower calcium content. The legume seeds, notwithstanding their high content of protein, are without any appreciable value for improving the diets which predispose to pellagra, because of the poor quality of their protein and their failure to supplement a diet derived from vegetable foods of the storage tissue class in other respects.

Both meats and eggs are more expensive sources of protection against faulty diet than milk. An effective campaign of education should be conducted in all districts where diets of a character likely to predispose to pellagra are common, informing the people about the great benefits to health from regular and very liberal use of leafy vegetables. This would be a movement toward the establishment of dietary practices resembling those of the more nearly vegetarian groups of Chinese and Japanese, and if in addition the inclusion of a suitable amount of milk in the diet can be secured,

not only would pellagra disappear, but the general health of the people would be promoted.

The prevalence of pellagra in certain parts of the South rather than in other sections of the country is probably closely connected with the development of the modern milling industry. This places in the grocery store the degerminated and decorticated part of the grain. The rise of the sugar industry offers for human consumption both sugar and molasses in quantities unheard of until recent years.

The widespread practice of growing a cash crop (cotton), and of depending on the retail store for the greater part of the food supply

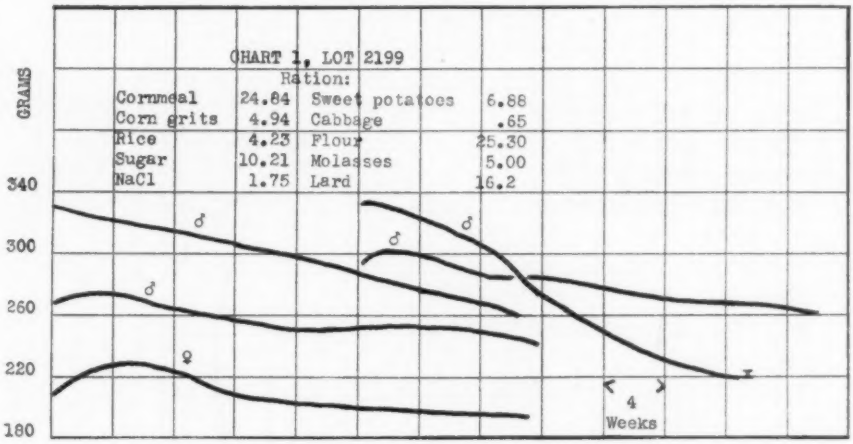

CHART 1.

rather than of engaging in diversified farming appears to be in great measure responsible for the existence of pellagra. The food products which can be handled commercially without hazard are not in general satisfactory foodstuffs unless properly supplemented with certain others which correct their deficiencies.

Chart 1.—Lot 2199 illustrates the failure of adult rats to maintain normal nutrition on a diet closely similar to that employed by Goldberger in his attempt to produce experimental pellagra in man. The only difference was that he employed cabbage, turnip top greens, and collards, whereas we replaced the two latter leafy vegetables by cabbage, but the total dry matter derived from a leaf source was the same in both diets.

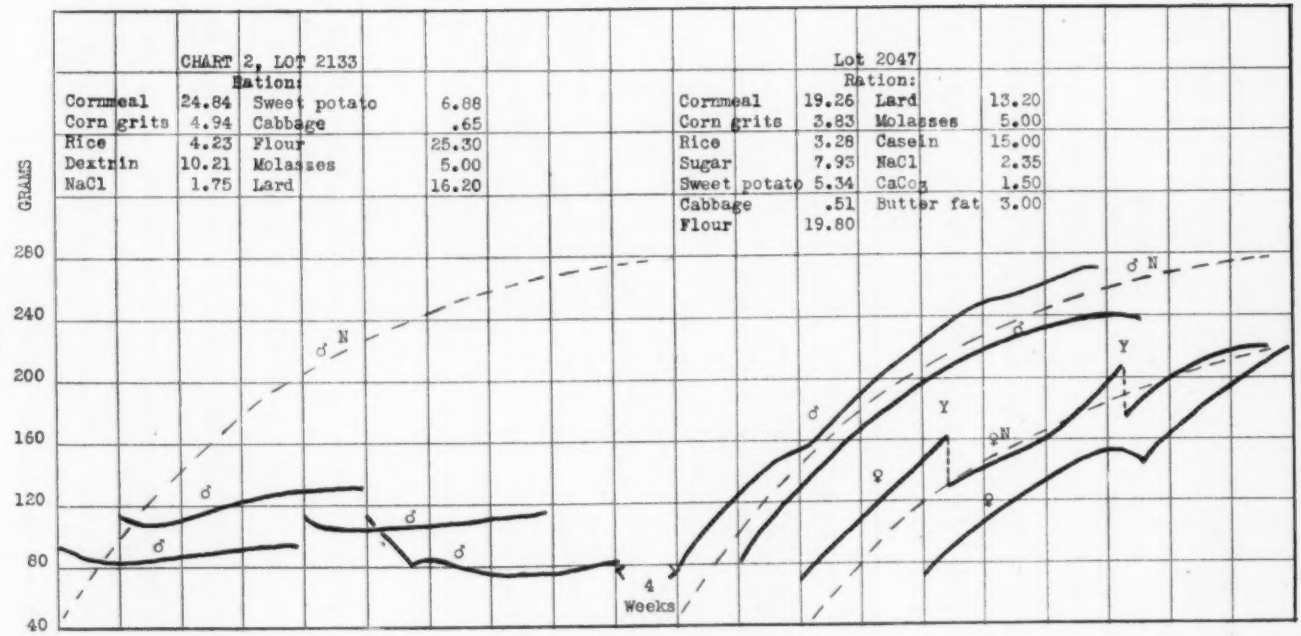
CHART 2, LOT 2133
Ration:
Cornmeal 24.84
Corn grits 4.94
Rice 4.23
Dextrin 10.21
NaCl 1.75
Sweet potato 6.88
Cabbage .65
Flour 25.30
Molasses 5.00
Lard 16.20
Lot 2047
Ration:
Cornmeal 19.26
Corn grits 3.83
Rice 3.28
Sugar 7.93
Sweet potato 5.34
Cabbage .51
Flour 19.80
Lard 13.20
Molasses 5.00
Casein 15.00
NaCl 2.35
CaCo3 1.50
Butter fat 3.00
GRAMS
280
240
200
160
120
80
40
♂ N
♂
♀
Y
♀N
4 Weeks

CHART 2.

Judging from such studies as we have made it appears that all leaves have much the same dietary properties, except such as contain obnoxious or poisonous products.

This diet afforded wide variety, palatability, and as far as chemical analysis could show, a satisfactory composition in every respect, but it proved unsatisfactory because it was derived too largely from the group of plant products which are functionally storage tissues. Less than 3 per cent of the dry matter of the diet was derived from leaf.

At the end of the feeding period these animals looked very old and rough haired, but were not emaciated. They were then about 16 months old, or somewhat less than one-half through the normal span of life, which is about 36 months. The diet, therefore, failed to maintain for long the characteristics of youth. The animals did not show any signs of a "deficiency" disease as beri-beri or xerophthalmia, for no specific lesion could be detected. There was nothing suggestive of the lesions seen in pellagra. The skin appeared healthy, and there was no diarrhea or abnormal appearance of the mucosa. It is certain that no combination of products derived from the endosperm of seeds, together with tubers and edible roots, will form a satisfactory food mixture for the support of growth in the young or of satisfactory nutrition in the adult. It seems reasonable to suppose that animals restricted to this type of diet would be rendered susceptible to infections of low order of virulence, against which well nourished individuals would be immune.

The cabbage, beets, turnips, and potatoes employed were dried at about 70°C. and ground. The potatoes were thoroughly steamed before drying.

Chart 2.—Lot 2133 shows the failure of young rats to grow on a diet which was similar in composition to that employed by Goldberger in his efforts to produce pellagra experimentally in man (15), except that cabbage was the only leaf product, and dextrinized starch replaced cane sugar. It consisted of corn-meal grits, rice, dextrin, bolted wheat flour, molasses, sweet potato, cabbage, baking powder, and lard.

The animals confined to this diet failed to grow, but in most cases were able to maintain their initial body weight for a period of nearly 4 months. The condition of the skin remained normal.

There was none of the roughness or soreness which we have frequently seen in rats long confined to certain types of faulty diets (3). They became thin and frail, but presented none of the symptoms which were observed by Chittenden and Underhill in dogs as the result of restricting them to a diet of peas, crackers, and cottonseed oil. That this diet is deficient only in respect to well recognized dietary factors is shown by the behavior of Lot 2047, which was fed essentially the same mixture supplemented with purified protein, inorganic salts, and butter fat (fat-soluble A). With these additions the diet is rendered sufficiently complete to make it possible for young rats to grow at nearly the normal rate to what may be regarded as but little below the normal adult size, and to produce young. They were not able to rear their young, and cannot, therefore, be considered to be in an entirely normal state of nutrition.

Lot 2047 shows the growth curves of a group of young rats which were fed a diet which was identical with that of Lot 2199, Chart 1, except that the former was supplemented with purified protein, inorganic salts, and fat-soluble A. These additions rendered the diet very nearly complete, since the animals were able to grow at a good rate and to produce young. They remained somewhat undersized in all cases. Although one female remained sterile, the other produced two litters of young but was unable to rear them. It is not entirely clear what was the limiting factor in causing these animals to fall below satisfactory nutrition. It may well be that the preponderance of products of the endosperm of seeds made the phosphorus content of the diet too low. We have not yet determined whether this is the case. It is also probable that when such a high percentage of the diet is derived from degerminated and decorticated seed products, starch, sugar, and molasses, the content of the dietary factor water-soluble B is near the actual amount required for the maintenance of growth, and is below the amount necessary to promote optimum well being. All animals were supplied with iron in the drinking water, and once each week with iodine, according to the usual routine procedure in our colony.

Chart 3.—Lot 2067 shows the slow rate of growth and early stunting of young rats which were fed a diet containing the entire substance of three kinds of seeds, *viz.*, peas, rolled oats, and navy

beans; the portion representing the endosperm of three seeds (bolted wheat flour, degerminated corn-meal, and polished rice), together with 10 per cent of the solids of the diet derived from round steak. This diet furnished 19.4 per cent of protein, wide variety of origin, palatability in high degree, and was derived from both animal and vegetable foods, yet it was not of a character suitable for the maintenance of satisfactory nutrition during growth, nor did it prevent the early aging of the animals. The animals in this group were very old and dirty looking at the age of 6 months. These results emphasize the point to which we have called atten-

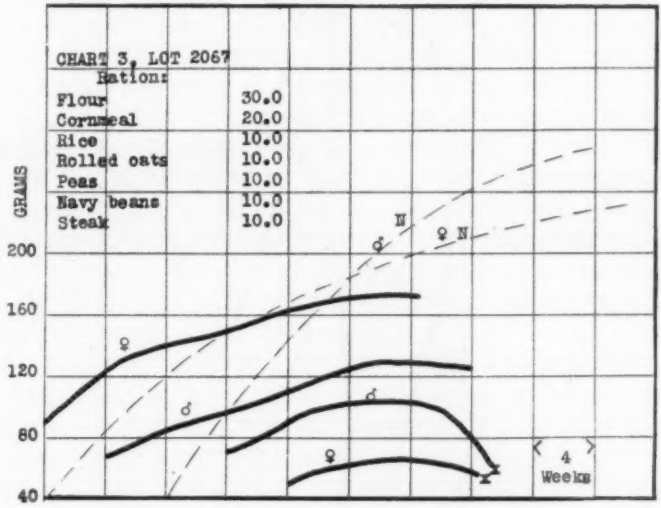

CHART 3.

tion on several occasions; *viz.*, that all the foods which were included in this diet are faulty in respect to two factors, one of which is the lack of sufficient calcium, sodium, and chlorine, the other a lack of a sufficient amount of the dietary essential fat-soluble A. These faults can be corrected by the inclusion in the diet of a sufficient amount of milk and eggs (see Chart 5, Lot 2148), and to some extent by the amounts of leafy vegetables which can be eaten by man. Eggs correct all faults in any diet except with respect to calcium, which element is not abundant in the contents of the shell. The growing chick derives a considerable amount of lime from the shell during the period of incubation.

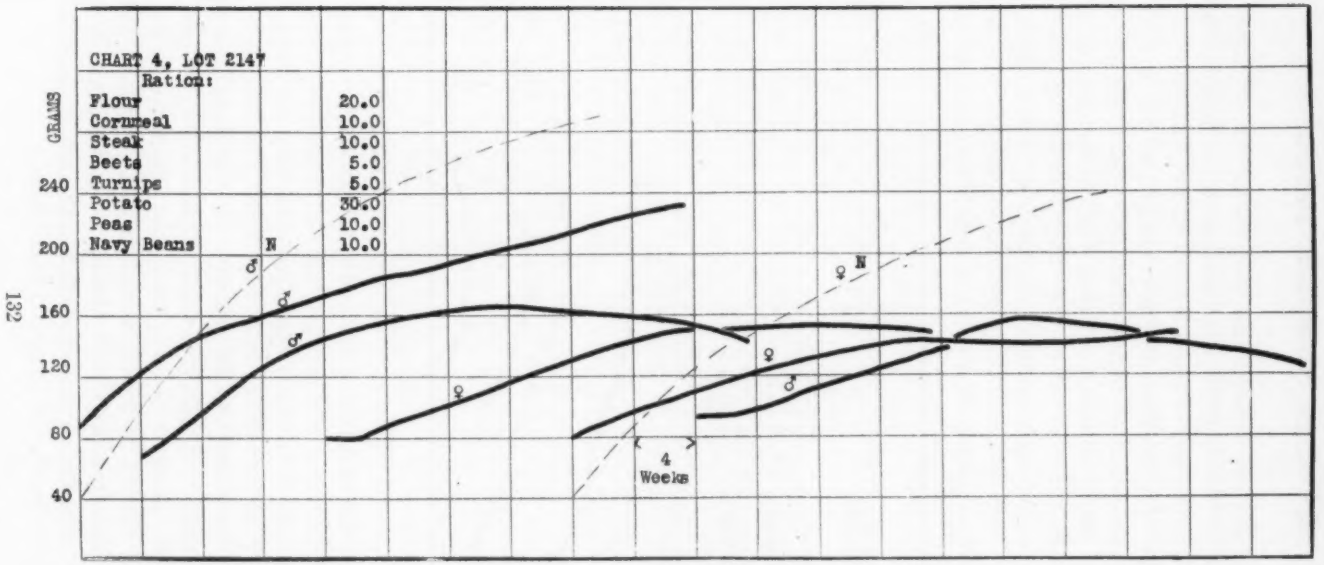
CHART 4, LOT 2147
Ration:
Flour 20.0
Cornmeal 10.0
Steak 10.0
Beets 5.0
Turnips 5.0
Potato 30.0
Peas 10.0
Navy Beans 10.0
GRAMS
240
200
160
120
80
40
N
♂
♂
♂
♀
♀
N
♀
♂
4
Weeks

CHART 4.

Chart 4.—Lot 2147 illustrates the growth of a group of young rats which were fed a diet consisting of two endosperm products (wheat flour and degerminated corn-meal), two edible roots (beets and turnips), one tuber (potato), two legume seeds (peas and navy beans), and 10 per cent of the dry matter of the diet as cooked, dry round steak. The important thing to note about this food mixture is that it is derived entirely from storage organs of plants or products made from them, together with muscle tissue. The latter is a highly specialized tissue, which does not manifest metabolic functions comparable with the glandular organs. Muscle tissue resembles the seeds in its dietary properties except that its proteins are of better quality. It does not supplement foodstuffs of the storage organ group except with respect to protein, and accordingly such a diet as is here described is deficient in two respects, *viz.*, in calcium, sodium, and chlorine among the inorganic elements and with respect to fat-soluble A.

These rats are too short and broad to be considered normal. They had begun to look old at the end of 160 days on this diet, and at the end of 10 months they were very thin haired and aged looking, with very scaly, sore ears and tails. The eyes have begun to show signs of inflammation and apparently they are on the verge of developing xerophthalmia.

A dinner consisting of steak, bread made without milk, butter, potatoes, peas or beans, gravy, a flavored gelatin dessert, and coffee, may appear to constitute a satisfactory meal, for it affords variety, palatability, and meets the requirements of the food chemist or the dietitian, but it would not promote health in an experimental animal over a very long period if taken as a monotonous food supply.

Chart 5.—Lot 2148 is of special interest when compared with Lot 2147, Chart 4. Their rations were closely similar, consisting of cereal products, legumes, tubers, and edible roots, but the ration of Lot 2148 contained 10 per cent of dried whole milk (Merrill-Soule). A comparison of the growth curves shows how marked is the tendency of milk to correct the deficiencies of foods of the storage organ group of vegetable origin, and likewise of muscle tissue.

These animals were able to grow at the normal rate to the full adult size, except one individual. Reproduction was distinctly

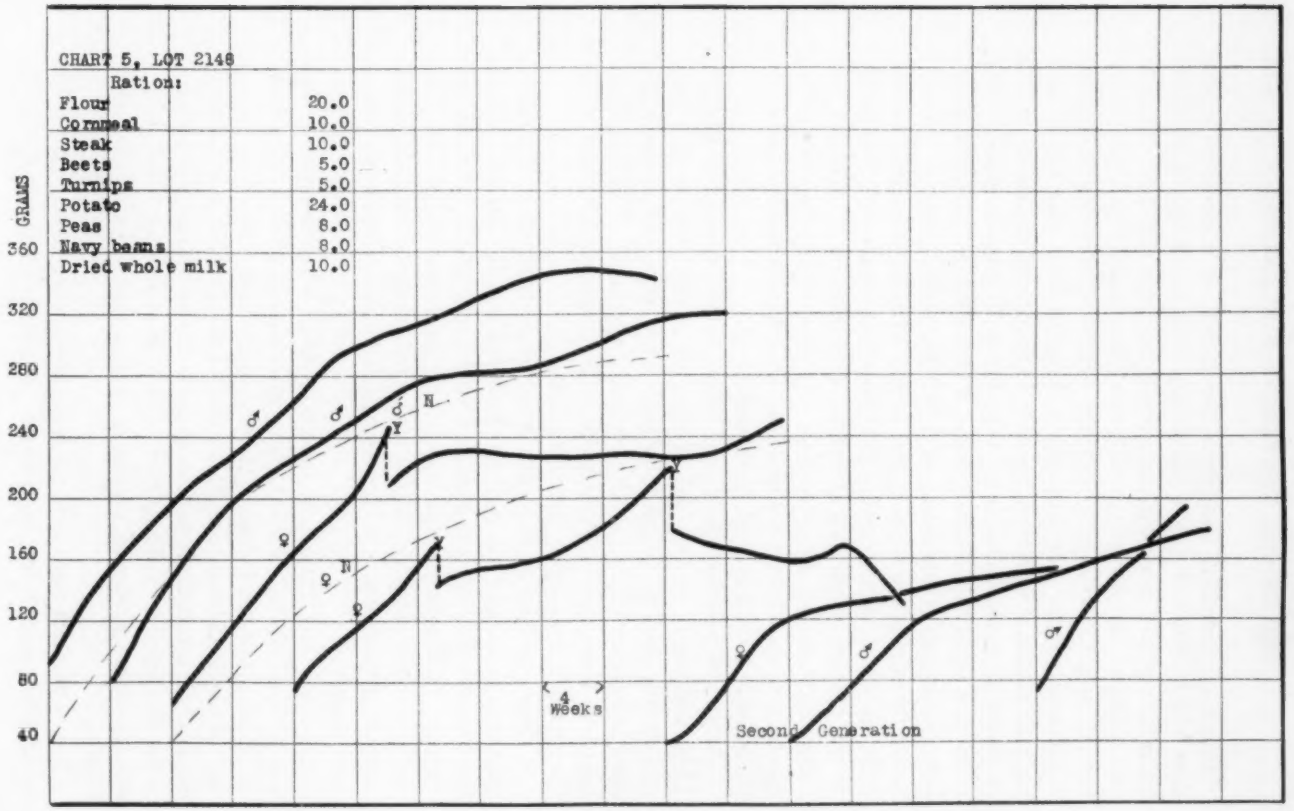
CHART 5, LOT 2148
Ration:
Flour 20.0
Cornmeal 10.0
Steak 10.0
Beets 5.0
Turnips 5.0
Potato 24.0
Peas 8.0
Navy beans 8.0
Dried whole milk 10.0
GRAMS
360
320
280
240
200
160
120
80
40
♂
♀
N
Y
4 Weeks
Second Generation

CHART 5.

below normal in amount, but the young which were born were reared with a fair degree of success. That the diet is somewhat faulty is shown by the reproduction records, and by the behavior of the second generation when confined to the diet of the parents. We have not yet determined the reason for the failure of 10 per cent of milk to supplement the remainder of the diet completely. It seems almost certain, however, that it is due to the failure of the amount of milk added, although liberal, to supply enough calcium. We have been able to relieve xerophthalmia in rats by feeding the fat isolated from evaporated milk (4), and have convinced ourselves that the fat of dried whole milk still contains a considerable amount of fat-soluble A, so a shortage of fat-soluble A cannot be the limiting factor in this case. We know that the proteins of this diet are of excellent quality, and the content of protein amounted to 18 per cent of the food mixture, which is an ample amount. Iron was furnished in the drinking water, and is abundant in red meats, and since the fault must be in the inorganic moiety, it is most probably calcium.

Chart 6.—Lot 2152 shows that a diet derived from the entire substance of three seeds together with polished rice, degerminated corn-meal, and bolted wheat flour, supplemented with calcium carbonate and sodium chloride, induced better growth than did the same seed products supplemented with 10 per cent of cooked, dried round steak (see Chart 3). It is evident that the lack of these salts is a matter of greater importance than is the quality of the protein, or the relative shortage of fat-soluble A. Meat enhances such a grain mixture only with respect to the improvement of the quality of the protein (see Chart 7).

Although the two females in the group produced two litters of young each, none were satisfactorily nourished. One female had a litter of nine which at 16 days weighed 117 gm. The number of young was reduced at that time to five. Four of these reached an age of 45 days at which time they weighed collectively 103 gm. Although very small for their age, they were active. A litter from another mother (six young) weighed 98 gm. at 19 days of age. Three reached the age of 34 days when they weighed 67 gm. together. Two reached the age of 69 days. They were then very puny and weighed together but 73 gm. They should have weighed at least 100 gm. each at that age. (Compare these repro-

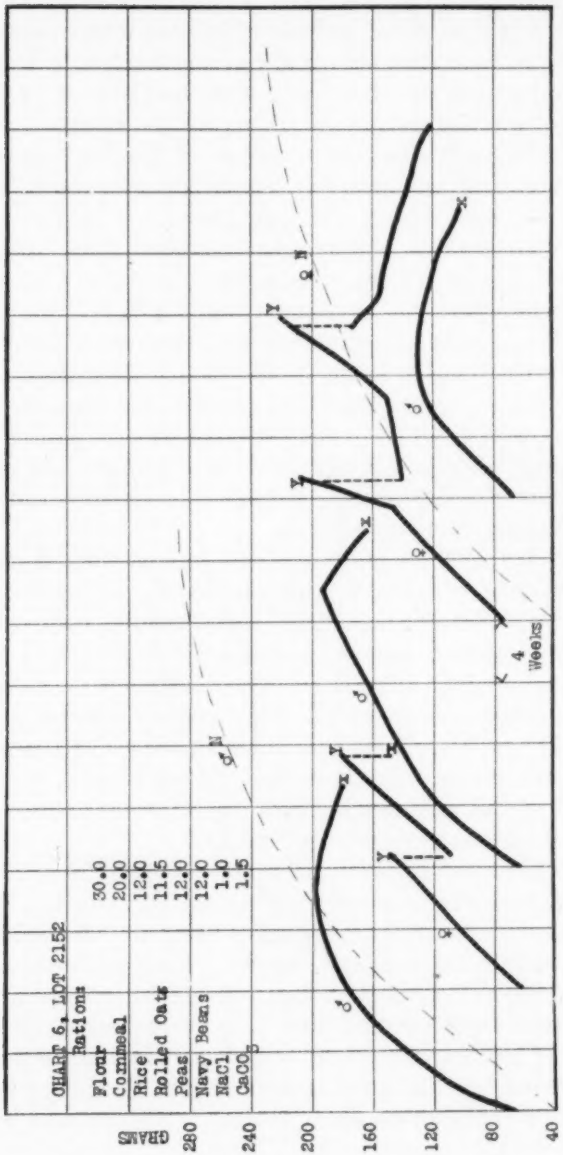
CHART 6. LOT 2152
Rations
Flour 30.0
Cornmeal 20.0
Rice 12.0
Rolled Oats 11.5
Peas 12.0
Navy Beans 12.0
NaCl 1.0
CaCO3 1.5
GRAMS
280
240
200
160
120
80
40
4 Weeks

CHART 6.

duction records with those of Charts 7, 9, 10.) At the age of 5 to 6 months the rats restricted to this diet were very old looking. Even with diets derived from wholesome natural foodstuffs, and having such a composition as will comply with the standards of the food chemist, the source of the several components of the food supply is a matter of the first importance, and is one of the principal factors in determining the span of life and vigor of an animal (compare with Chart 3, Lot 2067).

Chart 7.—Lot 2153 received a mixture which was similar in all respects to the ration of Lot 2067, Chart 3, but supplemented with sodium chloride and calcium carbonate. Thus supplemented, the mixture of seeds and seed products with 10 per cent of beefsteak supported good growth to the full adult size, and the females produced about the number of young which we should expect of well nourished rats. Two first generation females produced six litters (forty-four young), of which thirty-five were successfully weaned. The second generation did not do so well with their young, although the diet enabled them to grow up fairly well.

Although the young of the original experimental group were apparently in excellent condition, they were actually not so. One of these second generation females died at the age of 4½ months without having had young. Another grew up in a nearly normal way, had a litter of young at the age of 129 days, but they were all dead, and the mother died a month later. A third female appeared normal as she reached maturity, and had a litter of young when she was 87 days old. The young were small and puny and were eaten by the mother. The diet sufficed for the maintenance of the original group during the growing period, and supported fair vigor during reproduction, but there were sufficient faults in its make-up to undermine the vitality of the offspring. Such records as these convince us that we have now refined our experimental technique so as to make possible the detection of very slight faults in the diet.

The practice of indulging in a variety of foodstuffs, and of alternating among certain of them instead of restricting the diet to a monotonous mixture is without doubt a highly desirable one, but where the selection of food is limited too largely to that group of products which are functionally storage tissues of plants and to meats (muscle tissue) variety within this range will not prevent deterioration of the race.

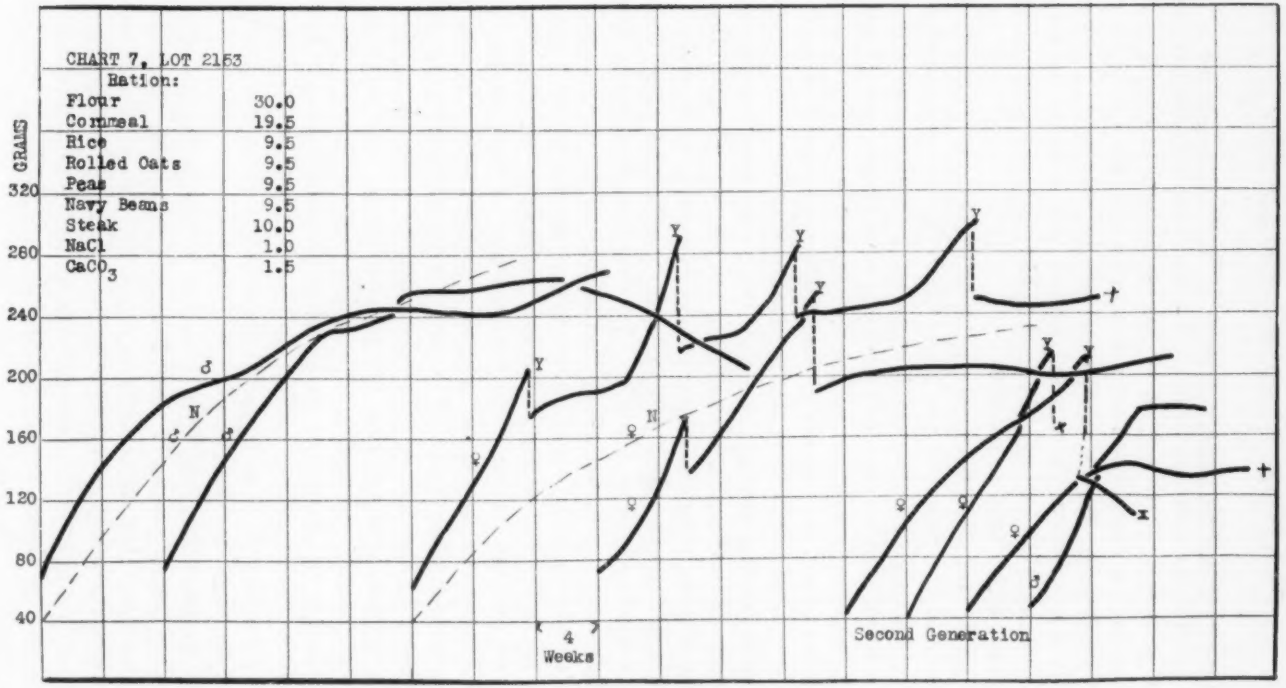
CHART 7, LOT 2153
Ration:
Flour 30.0
Cornmeal 19.5
Rice 9.5
Rolled Oats 9.5
Peas 9.5
Navy Beans 9.5
Steak 10.0
NaCl 1.0
$CaCO_3$ 1.5
GRAMS
320
280
240
200
160
120
80
40
4 Weeks
Second Generation

CHART 7.

Chart 8.—The records of Lot 2064 show to what degree a content of cabbage leaf equivalent to 10 per cent of the diet supplements a mixture of wheat flour, corn-meal, potato, peas, navy beans, and beefsteak. Without the presence of the leaf in this diet very little growth could be secured. With 10 per cent of cabbage growth could take place, but the ultimate size attained was not much greater than half the normal adult size. Two females each had a litter (six and seven young respectively) of which eleven

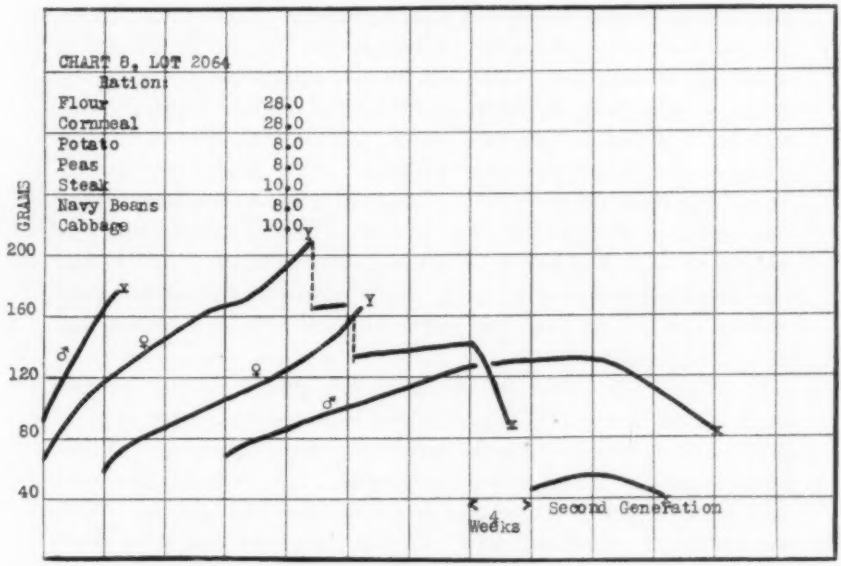

CHART 8.

were weaned but none of them was normal. These were too short, an appearance which is characteristic of many young rats in our colony which become somewhat deformed because of malnutrition. We are studying this condition closer. They were very nervous and undersized. Five young in one litter weighed 204 gm. at the age of 49 days. Six young in the other litter weighed 187 gm. at the age of 46 days. We have seen a litter of four well nourished young rats reach a collective weight of 300 gm. at the age of 35 days.

When the rats on this ration had been confined to the diet 5½ months, they were already very old and rough, and their condition grew worse rapidly, none living beyond the age of 8 months. The offspring were unable to grow when confined to the diet of their parents.

In order to appreciate the extent to which the addition of moderate amounts of leaf enhances a diet derived from seeds, tubers, roots, and meats, Lot 2064 should be compared with Charts 3 and 4. A comparison of Charts 3 and 4 with 6 and 7 illustrates the good effects of supplementing this type of diet with sodium chloride and calcium carbonate. A comparison of the physiological results of feeding a diet derived from products belonging to the storage organ group in plants and the same mixture supplemented with milk is seen in Charts 4 and 5. Chart 7 shows, when compared with Chart 8, that the correction of the inorganic factor is one of the chief values of the inclusion of a leaf in the diet. That the addition of leaf enhances a diet of seeds, meat, and the necessary salts is shown by a comparison of Charts 7 and 9, and the desirability of having milk, a leaf, and likewise additions of sodium chloride and calcium carbonate is shown by a comparison of Charts 9 and 10.

Chart 9.—The ration of Lot 2069 was similar to that of Lot 2148, Chart 5, except that milk was replaced by 10 per cent of dried cabbage and sodium chloride and calcium carbonate. These animals grew well and were very prolific. Two females produced seven litters (thirty-six young) up to the age of 8⅓ months. This we regard as normal fertility. Of the thirty-six young by these two mothers twenty-eight were successfully weaned. A daughter of one of them, when confined to the diet of the group, has had four litters (twenty-one young) at the age of 259 days. The first litter was born when she was but 71 days old, which shows that she reached maturity early. She reared thirteen of the twenty-one young, but these were too short for their age and weight, a condition frequently seen in stunted rats. One third generation female (granddaughter of the first group) had her first litter of four young at the age of 94 days. These weighed collectively 202 gm. at the age of 28 days, a weight which is highly satisfactory. At the age of 11 months the original group look very old and rough, and are distinctly poorer in condition than is Lot 2070,

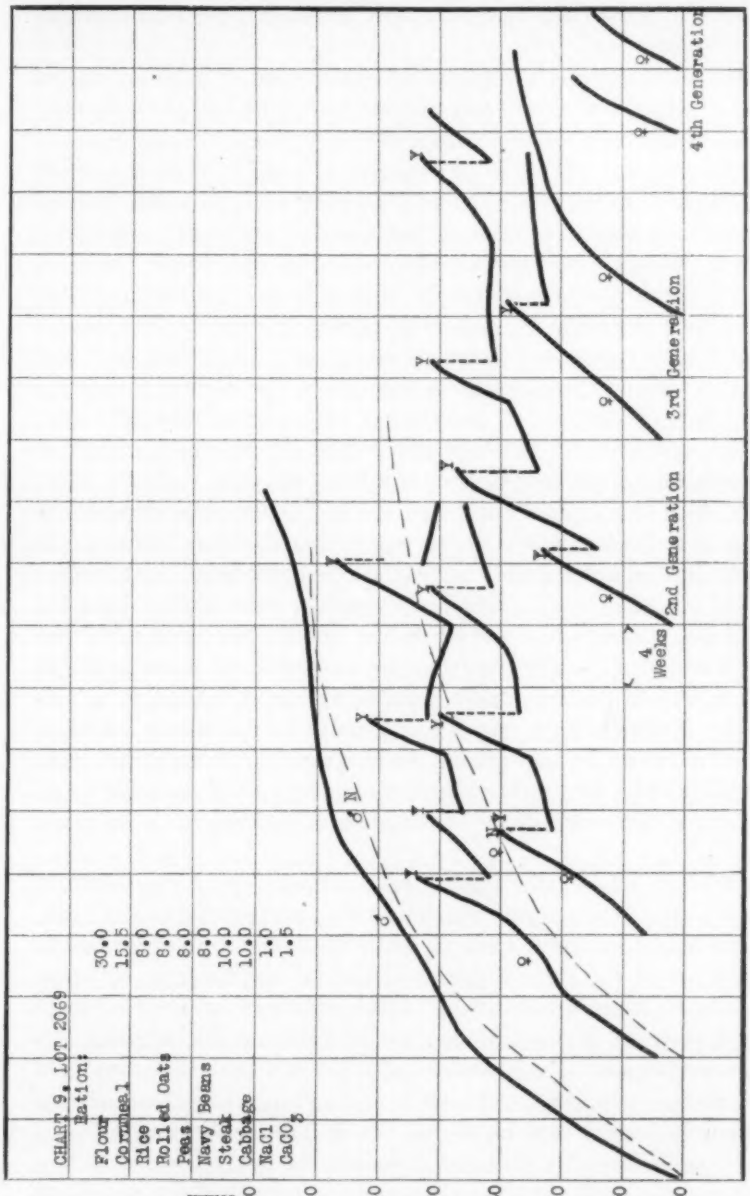
CHART 9. LOT 2069
Ration:
Flour 30.0
Cornmeal 15.5
Rice 8.0
Rolled Oats 8.0
Peas 8.0
Navy Beans 8.0
Steak 10.0
Cabbage 10.0
NaCl 1.0
CaCO3 1.5
GRAMS
320
280
240
200
160
120
80
40
4 Weeks
2nd Generation
3rd Generation
4th Generation

CHART 9.

Chart 10, whose diet is similar, but contains 5 per cent of whole milk powder.

This data reveals the profound importance of a knowledge of the dietary properties of our natural foodstuffs and the combinations which have efficient supplementary relations from the dietary standpoint. This is very nearly the same type of diet which is common among many Oriental peoples, with the differences of course that they have more or less variety in the leafy vegetables which they eat, but restrict themselves to rice as the principal cereal, employ soy beans instead of navy beans, and use more fish than flesh of mammals, especially among the poor. The important thing to appreciate is the classification of their foods and ours on the basis of their biological function. The dietary properties can then, in the light of our studies with several natural foods, be interpreted in a manner that surpasses the most sanguine expectations of the investigator of a few years ago.

It should be kept in mind that the leaf is not the equivalent of milk as a supplementary and corrective food for diets largely made up of degerminated and decorticated cereal products, peas, beans, rolled oats, and steak. The leafy vegetables are highly desirable additions to the diet and their use should be considerably extended in this country, but they are not sufficiently acceptable to the palate of man to make possible the consumption of a sufficient amount of this class of foods except by one who is schooled, as are many of the poor among the Orientals, in eating for the sake of maintaining life, rather than for the pleasure of tasting a great variety of dishes which are the creation of geniuses in the culinary art.

Chart 10.—Lot 2070 illustrates the growth and reproduction records of a group of rats which was fed a similar diet to Lot 2148, Chart 5, and Lot 2069, Chart 9, but with the mixture of foodstuffs derived from the storage tissues of plants, and the muscle tissue of animals supplemented with both 10 per cent of dried cabbage and 5 per cent of dried whole milk, and sodium chloride and calcium carbonate. Even when a diet derived from seed products and meat is supplemented with liberal amounts of leaf, which improves its quality in some degree, it still falls far short of the optimum, as is shown by the great improvement in vitality and ability to meet the more strenuous demands of reproduction, to escape

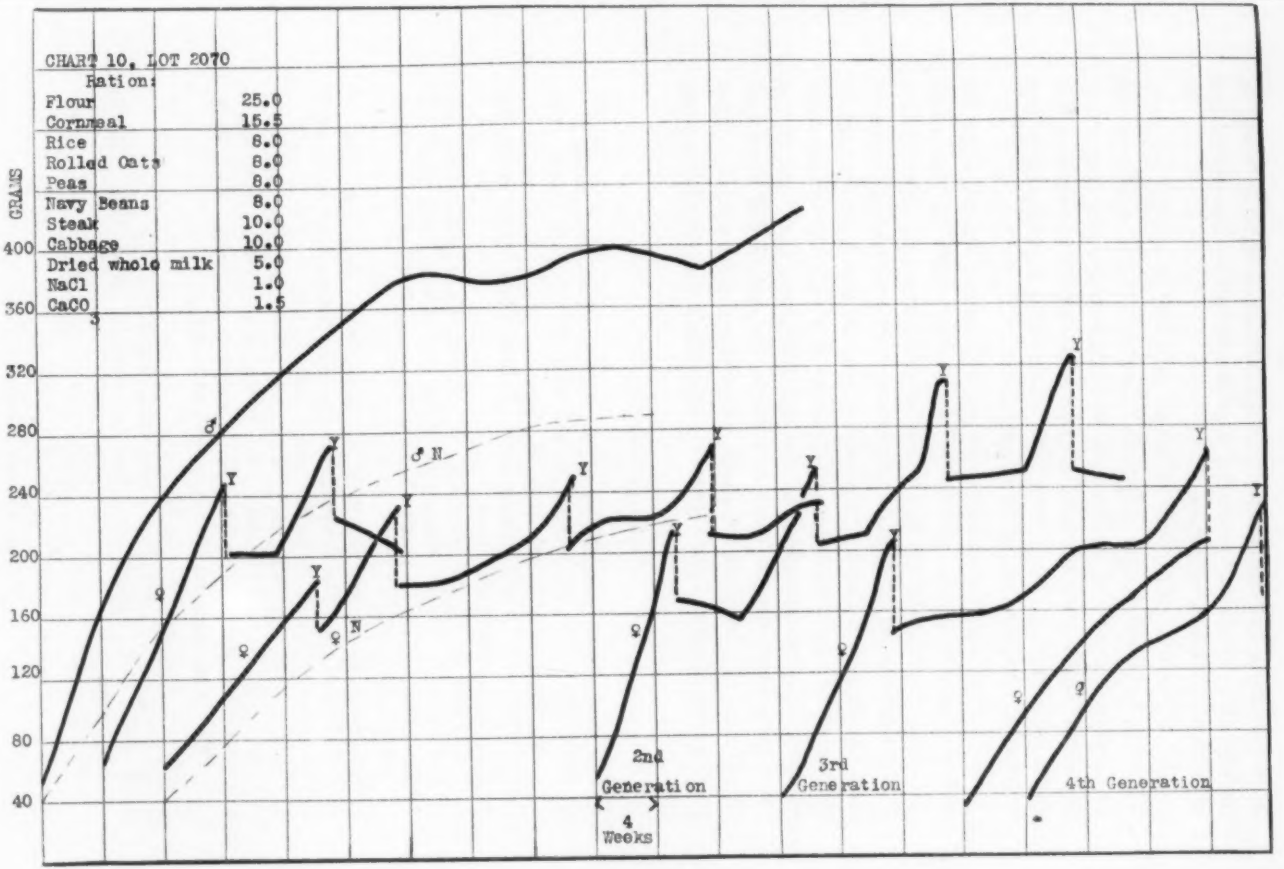
CHART 10, LOT 2070
Ration:
Flour 25.0
Cornmeal 15.5
Rice 8.0
Rolled Oats 8.0
Peas 8.0
Navy Beans 8.0
Steak 10.0
Cabbage 10.0
Dried whole milk 5.0
NaCl 1.0
CaCO3 1.5
GRAMS
400
360
320
280
240
200
160
120
80
40
♂
♀
♂ N
♀ N
Y
2nd Generation
4 Weeks
3rd Generation
4th Generation

CHART 10.

lung infection, and to maintain to an advanced age the vigor of middle life. Such improvement can be secured by the liberal use of milk with diets of this type.

The reproduction records of these rats are of special interest. Those in Lot 2069, Chart 9, had an infant mortality of 22 per cent in the first generation on the diet, and the female from this group of young which grew up on the same diet lost 38 per cent of her twenty-one young. It is very rare indeed that a young rat is not successfully weaned in our breeding stock.

An idea of the infant mortality in well nourished rats can be gained from the records of Lot 2070. In the original group in Lot 2070 the two females up to the age of 339 days had seven litters (forty-eight young) of which forty-seven were successfully weaned. Two females from these young which grew to maturity had up to the age of 284 days six litters (50 young) of which forty-eight were weaned. A granddaughter of the original group (*i.e.*, one of the third generation on the diet) had up to the age of 259 days had two litters (17 young) of which all were weaned successfully. Two great granddaughters each weaned successfully a litter of 8 young.

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EXPLANATION OF PLATE 1.

FIG. 1. Female rat from Lot 2147, Chart 4. The diet of this group consisted of wheat flour 20, degerminated corn 10, cooked, dried potato 30, peas 10, navy beans 10, beets 5, turnips 5, and cooked, dried beefsteak 10 per cent.

This diet was derived like that of Lot 2148 from degerminated cereal products, legume seeds, tubers, edible roots, and meat (muscle tissue), but differed from it in not containing milk. It afforded wide variety, had an appropriate chemical composition in so far as analysis could show, was palatable, and included only natural food products of recognized wholesomeness, and from both animal and vegetable sources. Notwithstanding these facts the nutrition of the animals restricted to this food supply was very faulty. The photograph shows a rat at the age of 308 days, or at about the end of the first quarter of the normal span of life for a well nourished animal of this species. It weighed but 137 gm. as compared with the one pictured from Lot 2148, whose weight was 245 gm. The pronounced stunting and very rapid aging of this group emphasize the importance of including in the diet the foodstuffs which we have designated as "protective foods." Milk is the most effective of these.

FIG. 2. Female rat from Lot 2148, Chart 5. The diet of this group consisted of bolted wheat flour 20, degerminated corn-meal 10, potato 24, peas 8, navy beans 8, turnips 5, beets 5, beefsteak 10, and dry whole milk (Merrill-Soule) 10 per cent.

This diet was derived from the same foodstuffs as that of Lot 2147, and in closely similar amounts, and differed from it only in containing 10 per cent of its dry substance as dry whole milk. The addition of this amount of milk corrected the faults in the remainder of the diet in a manner that is most surprising (see Charts 4 and 5). The animal was 308 days old and weighed 245 gm. when photographed. It was, therefore, but little beyond one-fourth through the normal span of life for the species.

FIG. 1.

FIG. 2.

(McCollum, Simmonds, and Parsons: Pellagra-Producing Diets. VI.)

IS LACTALBUMIN A COMPLETE PROTEIN FOR GROWTH?

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(From the Research Laboratory of Parke, Davis and Company, Detroit.)

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Recent reports of investigations suggest that there is a difference of opinion as to the growth-promoting value of lactalbumin. We are especially interested (1) in this point since many of our findings as to the relative value of certain vitamine preparations, when added to an otherwise complete diet, have been based upon the assumption that our basal protein, lactalbumin, is capable of promoting growth; that is, that it contains the fundamentals for furnishing *all* the protein complexes required.

Osborne and Mendel (2) have shown very clearly that they were able under their conditions to obtain excellent results with lactalbumin as the primary protein in their diets. In fact, they (3) found in comparing the food value of edestin, casein, and lactalbumin for the growth of young rats that "the superior efficiency of the lactalbumin in the nutrition of growth is demonstrated beyond question." "Thus, to produce the same gain in body weight, 50 per cent more casein than lactalbumin was required, and of edestin nearly 90 per cent more." In a recent article, Osborne, Mendel, and Ferry (4) suggested a method of calculating the growth-promoting value of various proteins in rations. This method was based upon correlating the gain in body weight with the grams of both total food and protein consumed. When the authors compared their results for casein and lactalbumin on this basis, they found that the same gains for these two proteins could be made, over the same length of time, only when the casein-fed rats consumed 20 and 24 per cent more of ration and protein respectively than did the lactalbumin-fed rats.

On the other hand, McCollum, Simmonds, and Parsons (5) have taken the stand that lactalbumin is an incomplete and poor

protein for growing rats. Their conclusion was based upon the fact that when they compared casein with lactalbumin in two rations which were alike in all other respects, normal growth resulted in the first instance and almost no growth in the latter instance. Their basal diet was made up of peas 45 per cent, salt mixture 1.8 per cent, agar-agar 1 per cent, dextrin 38 per cent, and butter fat 5.0 per cent. 9 per cent of lactalbumin and of casein was added, respectively, to two portions of this basal ration, bringing the total protein up to about 18 per cent. The Osborne and Mendel rations consisted of 18 per cent protein (lactalbumin or casein), 28 per cent protein-free milk, 28 per cent starch, 18 per cent butter fat, and 10 per cent lard. McCollum and his associates stated:

> "We are forced to the conclusion that lactalbumin is a poorly constituted or an incomplete protein and that the excellent results of Osborne and Mendel were due to the high proportion of nitrogen derived from the 'protein-free milk' which was present in their food mixtures and served to supplement the lactalbumin with respect to some as yet undetermined cleavage product which is essential for growth."

According to Osborne and Mendel, their protein-free milk was made up of 80 per cent lactose, 15 per cent inorganic salts, 2.2 per cent protein, 2.7 per cent non-protein matter, and carried the water-soluble B vitamine. Mitchell and Nelson (6) found that they could reduce the per cent of protein nitrogen in the "protein-free milk" by neutralizing the boiling milk whey; but by this procedure they contaminated the lactalbumin, since calcium phosphates were thrown down at the same time. Kennedy (7) has shown that the Osborne and Mendel protein-free milk contains "either unprecipitated protein or peptids of considerable size;" that about half of the nitrogen was precipitated by certain salts in the non-amino form, and that after tryptic digestion, there was an increase in amino nitrogen.

From the above facts, it is evident that protein-free milk is a complex mixture and possibly capable of carrying the proper kind of protein or nitrogenous cleavage products to furnish the nitrogenous supplement that may be needed for an incomplete protein. Whether there would be a sufficient *amount* of this nitrogenous supplement present is another question.

DISCUSSION.

In using lactalbumin as the sole protein in the diet of young rats and as a supplement to an incomplete protein for growth, we have obtained results which agree with Osborne and Mendel (Charts 1, 2, and 3). On the other hand, we have recorded data with lactalbumin which correspond to what McCollum, Simmonds, and Parsons found (Chart 4). That is, we have secured in many cases normal growth, and then again, in using the same lactalbumin in the same amount, we have obtained almost no growth at all. However, the rations differed in this respect—when protein-free milk was present we obtained growth, but when a vitamine extract, salt mixture, and starch were substituted for the protein-free milk, poor growth resulted. In other words, our results agreed with what McCollum and his associates claimed; *i.e.*, the protein-free milk was responsible for supplying the needed accessory for the lactalbumin. *Our results do not show that the nitrogenous matter in this protein-free milk played the fundamental part in furnishing the necessary supplement.*

According to Osborne and Mendel, the protein of their protein-free milk constituted about 0.13 per cent of the food. Mitchell and Nelson claimed that the reduction in nitrogen which they were able to effect was due to protein. If this is so, their procedure gave a still lower protein content than that of Osborne and Mendel, bringing it down to about 0.08 per cent of the ration. Such small amounts of protein as these would hardly seem to be sufficient to supply enough cleavage products to cause the difference that McCollum and his associates found between casein and lactalbumin, or that we have found (Charts 1 and 4). Our lactalbumin was separated out, in accordance with the method of Osborne and Mendel. It was, however, thoroughly digested with hot alcohol a number of times, and finally with ether or acetone. In this way, we considered that all traces of the water-soluble B and fat-soluble A vitamines were removed.

With the idea of determining whether this growth-promoting accessory factor was specific for the protein-free milk when lactalbumin was the sole protein, various rations were fed which differed only in respect to the kind of synthetic protein-free milk that was used. These were made up of the McCollum (8) salt

mixture 185, an equivalent amount of lactose that had been purified by repeatedly extracting a pure product with hot 95 per cent alcohol and drying in vacuum at a low temperature, purified starch, and an extract containing the water-soluble B vitamine. The extracts used were: a cold water digest of wheat germ; a hot alcohol (95 per cent) digest of wheat germ that had been previously extracted with ether to remove the oil; a cold alcohol (95 per cent) digest of dried brewers' yeast; or a water-soluble fraction of autolyzed brewers' yeast.

Chart 5 illustrates the growth-promoting property of these rations where lactalbumin was the sole protein and no protein-free milk was present, and shows that normal growth is possible. In the case of the water extract of wheat germ, which carried the vitamine B, the amount of solids used was based upon the nitrogen content, 5 per cent yielding 0.11 gm. of nitrogen per 100 gm. of ration. The hot alcohol (95 per cent) extract of the wheat germ carried 0.08 gm. of nitrogen per 100 gm. of ration. And the cold alcohol extract of dried brewers' yeast added 0.04 gm. of nitrogen per 100 gm. of ration. In the case of the hot alcohol extract of wheat germ and the cold alcohol extract of dried yeast, the amount of protein nitrogen must have been very low, if in fact any was present. Therefore, the amount of nitrogen that these two extracts added to the rations was in the non-protein form.

It would seem then, if lactalbumin were in reality an incomplete protein for growth, that the various extracts, furnishing the water-soluble B vitamine, carried the same non-protein cleavage accessory that the protein-free milk supplied. Further, the protein nitrogen, in the protein-free milk and in the water extract of wheat germ, was not concerned in supplementing the growth-promoting property of the lactalbumin.

McCollum and Davis (9) found that lactose and casein, unless thoroughly purified, seemed to carry enough water-soluble B and fat-soluble A accessories or vitamines to cause fairly good growth when these nutrients were added to a diet that was otherwise complete, except for these food hormones. Drummond (10) has confirmed their work. It might be contended, therefore, that these two accessory substances were adsorbed by our purified lactose and that they were responsible for the growth which we

obtained with the lactalbumin supplemented with the extracts of wheat germ and yeast.

Since both Osborne and Mendel, and McCollum and his associates used butter fat as their source of the fat-soluble A, we would be concerned only as to whether the *kind* or *source* of water-soluble B in the lactose was enough different from that in the wheat germ or yeast to be the contributing factor. The curves in Chart 6 show clearly that our lactose lacked a sufficient amount of the water-soluble B vitamine to promote growth. Thus, when this lactose and the lactalbumin were incorporated in rations, made up according to the Osborne and Mendel (18 per cent butter fat and 10 per cent lard) and the McCollum (5 per cent butter fat) formulas, the rats soon stopped growing and showed the usual symptoms which accompany a lack of this accessory in the diet.

As stated above, we have used rations which contained lactalbumin as the sole protein and which we considered from all the known facts obtainable to be abundantly capable of producing normal growth in rats. Yet some of these food mixtures gave poor growth (Chart 4) just as McCollum, Simmonds, and Parsons found with lactalbumin in their modified diet of peas, and as Osborne and Mendel (11) found when they compared the value of casein, lactalbumin, and edestin supplemented with their artificial protein-free milk and dried yeast. In these cases we did not use protein-free milk or lactose. When the rations were modified so that a part of the starch was replaced by an amount of purified lactose equivalent to that present in 28 per cent of protein-free milk, the rats began to grow normally (Chart 7).

From the evidence that we have offered, it would appear that the reason we were unable, in some cases (Chart 4), to obtain anything like good growth with lactalbumin as the sole protein, was due to the absence of lactose in the diets. Aside from this fact, it seems that the protein-free milk (which contained 80 per cent lactose) furnished nothing essential that was not supplied by the alcoholic extract of wheat germ or yeast, together with the synthetic salt mixture and lactose. Therefore, we do not agree with McCollum, Simmonds, and Parsons that the high nitrogen content of the protein-free milk contributed some cleavage product which adequately supplemented what they claimed to be incomplete lactalbumin. *Rather, we take the position that the lactalbumin*

is a complete protein in the sense that it is not lacking in any essential nitrogenous cleavage product for growth.

We are inclined to express the following tentative explanations, based upon our data, as to why lactalbumin does not produce normal growth unless supplemented by lactose in the form that we used it. First, the lactalbumin protein molecule may be so constituted that it is easily susceptible to toxic or inhibitory substances, while the casein protein molecule may be more stable or resistant. On this basis, we would say that in the wheat germ and yeast extracts, which carried the water-soluble B accessory, there may have been some toxic substance present which prevented normal growth with the lactalbumin until the lactose was introduced. With the modified lactalbumin-peas diet of McCollum, Simmonds, and Parsons, (5) there was also present a toxic substance which acted exactly as it did in our diets when no lactose was present, but in the casein-peas diet, the toxicity factor did not manifest itself.

Second, in the separation of the casein, the lactalbumin, and the protein-free milk from fresh skimmed milk, the lactose (and possibly the casein) adsorbs not only the water-soluble B and fat-soluble A accessories, as McCollum and Davis, and Drummond have shown, but it (and the casein) may *also* adsorb another as yet undefined water-soluble vitamine. In separating the lactalbumin, this accessory does not seem to be adsorbed, or if it is, the vitamine is split off much more easily during the subsequent washing and purification than is the case with lactose (or casein).

Applying this last suggestion, we would say that our purified lactose still had this undefined water-soluble vitamine adsorbed to it; on the other hand, that the casein of McCollum and associates was the carrier of this accessory; while in the feeding trials of Osborne and Mendel that this vitamine was present in their protein-free milk (2) but absent in their artificial protein-free milk without lactose (11). To substantiate this deduction regarding casein, we refer to the work of Funk and Macallum (12) who found that this protein, even though it was purified by the method which McCollum (9) uses, carried an accessory which they say may be analogous to the antiscorbutic vitamine.

It seems plausible to conclude from these data that the second explanation is the more probable, although it is easy to conceive

that both the above suggestions may be needed at times to explain the nutritive value of the lactalbumin. It is, therefore, difficult, in the light of these results, to agree with McCollum, Simmonds, and Parsons that the protein-free milk carried enough *nitrogenous* cleavage products to supplement what they designate as the incomplete and deficient growth-promoting lactalbumin, and yet when this protein-free milk supplemented the casein, it contributed nothing to accelerate the rate of growth of this protein and make it even the equal of the lactalbumin. If their conclusion was correct, how can one explain why Osborne and Mendel (2) obtained such contrasting results as to the relative growth-promoting values of lactalbumin, casein, and edestin when these proteins were incorporated in rations with the same amount of protein-free milk present in each (13)?

CONCLUSIONS.

1. Lactalbumin is a complete protein in the sense that it does not lack any of the nitrogenous cleavage products essential for growth. It can supplement a deficient growth-promoting protein (corn gluten) and, incorporated as the sole protein in a ration containing lactose, it produces a normal rate of growth, when present to the extent of only 10 per cent.

2. Lactalbumin is a protein which either is sensitive to certain toxic substances and under these conditions growth is retarded unless adequate adjustment is made in the diet; or it is a protein which does not appear to be able to adsorb what we tentatively designate as a vitamine (other than water-soluble B).

3. Lactose seems to be the essential constituent in the protein-free milk that contributes the accessory which makes an otherwise incomplete lactalbumin diet bring about normal growth in rats.

4. Lactose, when added to a lactalbumin diet, either may have the physiological property of overcoming the toxicity that inhibits growth taking place, or else it may carry a water-soluble vitamine (other than the water-soluble B) which appears to be essential to growth. This phase of the study is being continued.

The authors wish to express their appreciation to Lieutenant M. E. Slater for valuable assistance in the preliminary work of this

study. He was obliged to sever his connection with this work at an early stage as he was called into military service. To Mr. Charles Hunter also our thanks are due for aiding in the routine work.

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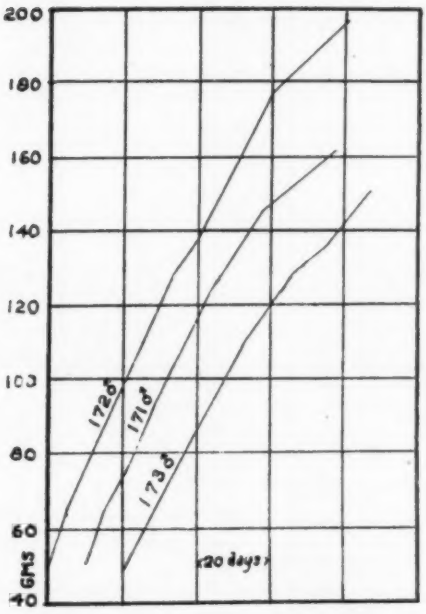

CHART 1. Good growth is obtained on a diet of 10 per cent lactalbumin protein, 28 per cent of Osborne and Mendel protein-free milk, 18 per cent butter fat, 10 per cent lard, and starch.

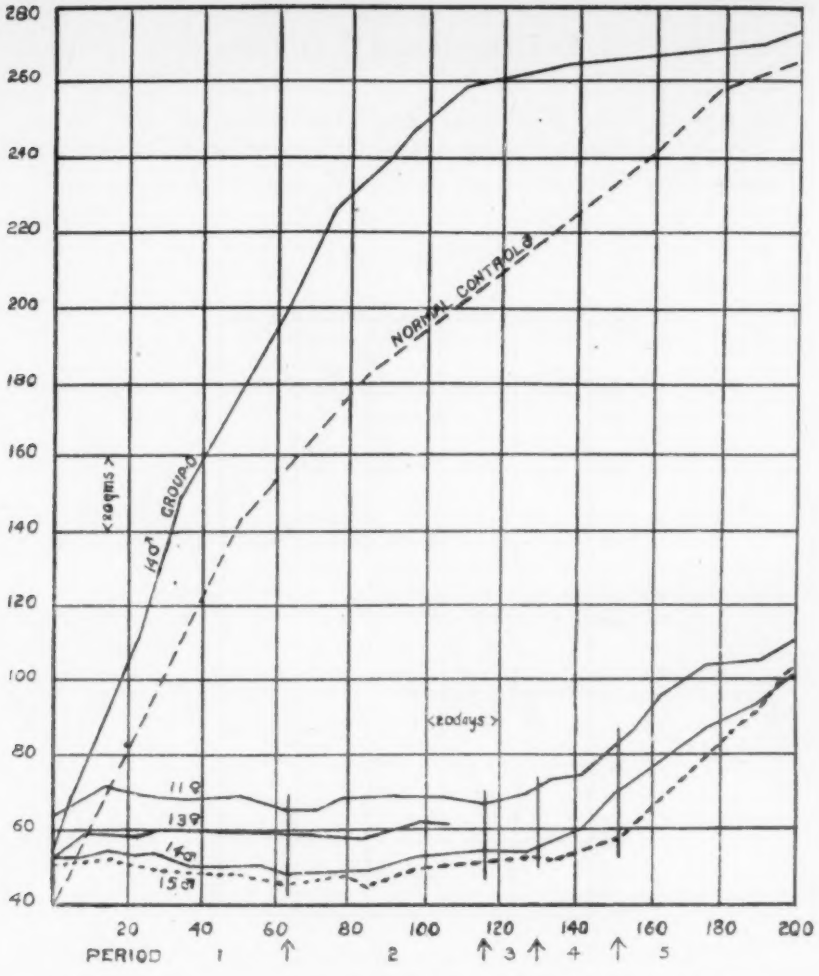

CHART 2. The curves illustrate in the case of Rat 14, Group O, the excellent growth response that can be obtained over a long period of time with the same diet that was fed to the groups of rats represented in Chart 1.

The curves also show, in the case of Rats 11, 13, 14, and 15, an interesting cycle of growth. Thus, throughout, the basal diet was 28 per cent of the Osborne and Mendel protein-free milk, 18 per cent butter fat, 10 per cent lard, and starch to make up the balance. In Period 1 (maintenance) there was 7 per cent corn gluten protein; in Period 2 (maintenance) 10 per cent corn gluten protein; in Period 3 (slight growth) 7 per cent protein of which 28 per cent was lactalbumin and 72 per cent corn gluten; in Period 4 (growth) 10 per cent protein with 18 per cent of it lactalbumin and 82 per cent corn gluten; and in Period 5 (growth) 10 per cent protein of which 32 per cent was lactalbumin and 68 per cent corn gluten. There can be no doubt but that the lactalbumin is capable of supplementing the growth-deficient corn gluten protein.

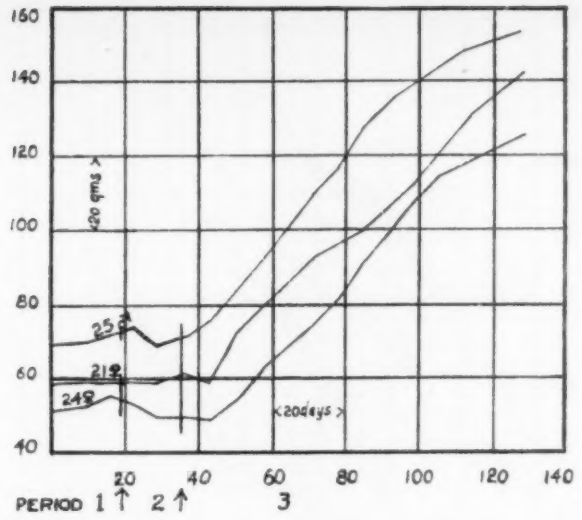

CHART 3. The curves illustrate the effect of incorporating the Mitchell and Nelson protein-free milk in place of the Osborne and Mendel product —the former being lower in protein nitrogen. Throughout, this protein-free milk was present. In Period 1 (maintenance) there was 7 per cent corn gluten protein; in Period 2, 6.5 per cent protein of which 22 per cent was lactalbumin and 78 per cent corn gluten; and in Period 3, 8 per cent protein of which 36 was lactalbumin and 64 per cent corn gluten. It will be seen that good growth took place in Period 3, when the lactalbumin supplemented the corn gluten to the extent of 36 per cent with the Mitchell and Nelson protein-free milk present.

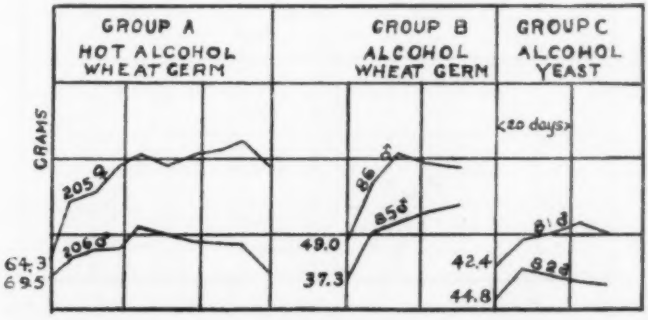

CHART 4. The curves illustrate the poor growth obtained with lactalbumin as the sole protein when protein-free milk was not used. The diet was made up of 10 per cent lactalbumin protein, 3.7 per cent salt mixture 185(McCollum), 18 per cent butter fat, 10 per cent lard, 5 per cent of the hot alcohol extract of ether-extracted wheat germ or 1 per cent of the cold alcohol extract of dried brewers' yeast, and starch to make up the balance.

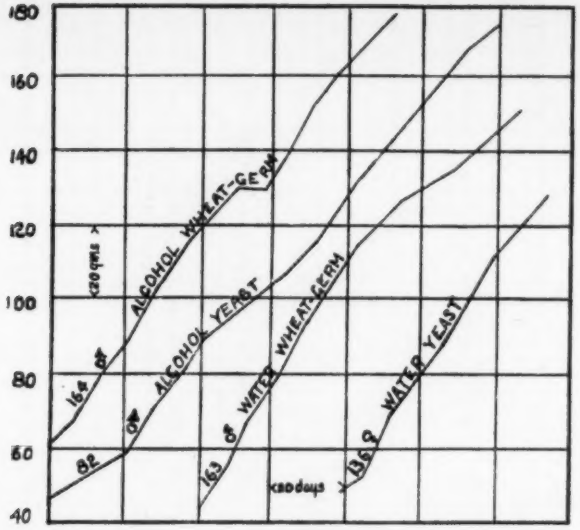

CHART 5. The rations fed these groups of rats were composed of 10 per cent lactalbumin protein, 18 per cent butter fat, 10 per cent lard, 3.7 per cent salt mixture 185 (McCollum), 24.6 per cent purified lactose, water and alcohol extracts of either wheat germ or yeast to supply the water-soluble B vitamine, and starch to complete the diet. Normal growth is possible with each of these four diets even in the absence of protein-free milk.

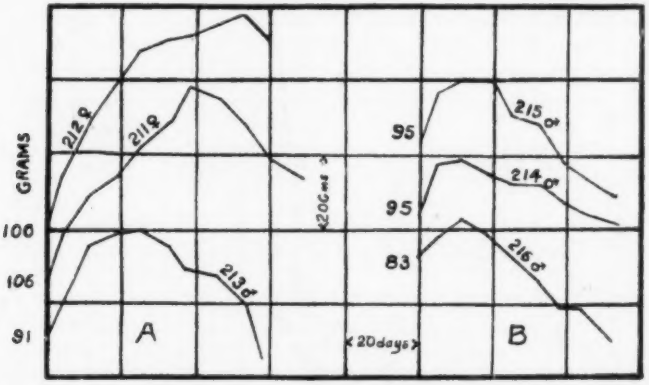

CHART 6. In these rations, the purified lactose was incorporated as the sole carrier of the water-soluble B vitamine. The diet for Group A was butter fat 5 per cent, lactalbumin protein 10 per cent, purified lactose 20 per cent, salt mixture 185 (McCollum) 3.7 per cent, and starch. The diet for Group B was the same as for Group A except that it contained less starch and more fat, butter fat 18 per cent and lard 10 per cent. The curves indicate that both the low and high fat diets are deficient—slight growth occurring at the start which is soon followed by a cessation of growth and subsequent decline.

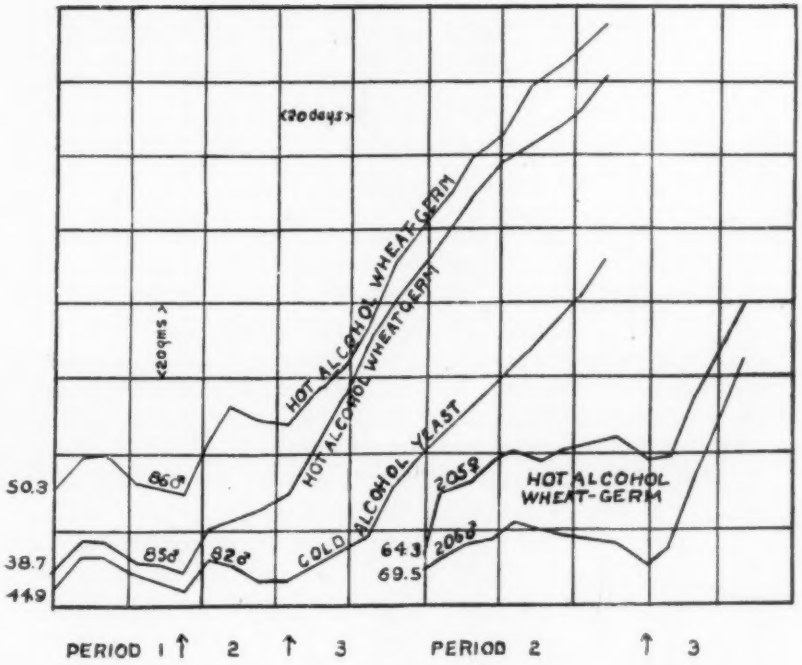

CHART 7. The curves show that lactose is an essential factor in the diet when lactalbumin is the sole protein. The basal ration throughout was lactalbumin protein 10 per cent, salt mixture 185 (McCollum) 3.7 per cent, butter fat 18 per cent, lard 10 per cent, and starch. The diet in Period 1 contained no water-soluble B accessory; in Period 2, this vitamine was supplied by an alcohol extract of wheat germ or yeast; in Period 3, 24.6 per cent of purified lactose replaced part of the starch of the ration used in Period 2. The growth response which followed the introduction of the purified lactose shows very definitely the importance of this constituent in these diets.

RELATIVE ABUNDANCE OF SERUM PROTEINS IN ALBINO RATS AT DIFFERENT AGES.

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Wells (1913) studied the relative abundance of serum proteins in rabbits as affected by age as well as by diet. Wells' observation is fundamentally important as the data not only reveal the influence of age, which is much neglected in such studies, but also form the basis for further experimental investigations. It appears desirable that the observation of Wells should be repeated or extended to other laboratory animals which are commonly used. I therefore have made a similar study on the serum of albino rats as these animals are extensively used and furthermore at The Wistar Institute rats from the colony of standard animals can be had for study.

The albino rats used in these experiments were fed on the ordinary laboratory diet and the blood was removed 24 hours after feeding. Suckling rats were isolated from the mother 8 to 16 hours before bleeding. The rats were etherized and bled directly from the carotid artery into a test-tube, care being taken not to sever the trachea or esophagus. The test-tube was closed to prevent evaporation and left at room temperature until the serum had separated. The blood was then centrifuged and the clear serum pipetted off and analyzed. In this work the method devised by Robertson (1915) was used; the total proteins were precipitated by 0.02 N acetic acid and the globulins by ammonium sulfate.

Altogether 66 specimens of serum, secured from 117 albino rats, were classified into fourteen groups according to age. In the majority of specimens, namely in 44, the desired amount of serum (1.5 to 2.0 cc.) was obtained from one rat, while in the younger rats, it was obtained by using from two to seventeen rats at once, the number increasing with decreasing age.

Refractive Index of Serum of Albino Rats at Different Ages.

Hatai (1918) found that the refractive index of the serum of albino rats varies clearly according to their age and in general increases with increasing age. Hatai noticed three distinct phases in the graph. The first phase is represented by the period of suckling, that is about the first 18 days, during which the refractive index rises rapidly. This period of rapid rise is followed by sudden fall of the graph, after which it rises again until the animal is about 70 to 90 days old. The end of this phase just precedes puberty. After 70 to 90 days of age the graph rises very slowly throughout the remainder of the span of life. The period of transition between the end of sexual maturity and the beginning of the adult phase is marked by irregularities in the graph, although no definite interpretation of this phenomenon has been given by Hatai.

For the rats employed in the present investigation I have also determined the refractive index of the serum. The results are given in Table I, Column 5, and also in the upper part of Chart 1.

Despite the fact of the small number (117) of rats examined by me, Chart 1 clearly shows in the upper graph the three phases mentioned by Hatai (1918). In my series, however, the end of both the suckling phase and the end of the puberty phase come several days later than in the records by Hatai; that is, at about 22 days for the former instead of 18 days, as shown by Hatai's rats, and for the latter at 85 instead of 70 days. This difference in the age at which the two critical phases occur is interesting because it is related to a period of poor nutrition in the rat colony in general, although the best animals obtainable were used for this work. It was noted at this time that the young were small for their age and required to be left with the mother longer than usual. The absolute value of the refractive index was also less than that found by Hatai.

Proteins of Blood Serum.

The results of the determinations of the proteins are shown in Table I, and the graphic representation is given in the lower part of Chart 1. A discussion of the changes in relative proportions of the serum proteins, shown in Chart 1, follows.

During the suckling phase, or for about the first 23 days, the percentage of total proteins and also of both albumin and globulins shows an increase with age. Reiss (1909) has also reported a similar increase of protein content in the human serum in the suckling period—after the period of very high content of protein which occurs during the first days of life in man.

From the end of the suckling period (23 days) to the age of 30 days, the percentage of globulins falls rapidly and then rises again

TABLE I.

Changes in Refractive Index as well as in the Proportional Amount of Serum Proteins in Albino Rats at Different Ages.

Age.	No. of samples analyzed.	Body wt. (average).	Body length (average).	N_D	Total proteins.	Albumins.	Globulins.	Non-protein substances.	Relative amount of	
									Albumin	Globulin.
days		*gm.*	*mm.*		*per cent*	*per cent*	*per cent*	*per cent.*		
Newborn.	1	—	—	1.34101	2.8	1.7	1.1	1.6	60.7	39.3
7	1	10.8	—	1.34095	2.9	1.6	1.3	1.5	55.2	44.8
14	2	18.3	82	1.34245	3.9	2.4	1.5	1.2	61.5	38.5
22	2	26.5	97	1.34399	4.4	2.3	2.1	1.6	52.3	47.7
30	5	24.7	96	1.34350	4.7	3.5	1.2	1.5	74.5	25.5
50	5	51.1	115	1.34508	5.0	2.8	2.2	1.5	56.0	44.0
80	12	80.8	144	1.34648	5.7	3.3	2.4	1.5	57.9	42.1
87	5	69.4	142	1.34694	6.2	4.0	2.2	1.3	64.5	35.5
94	7	76.8	146	1.34647	5.9	3.7	2.2	1.4	62.7	37.3
103	9	78.5	142	1.34745	6.2	3.8	2.4	1.5	61.3	38.7
140	4	130.8	171	1.34722	5.9	3.1	2.8	1.5	52.5	47.5
180	3	129.0	172	1.34700	6.3	4.2	2.1	1.3	66.7	33.3
305	6	177.9	191	1.34876	6.5	2.9	3.6	1.7	44.6	55.4
385	4	197.8	196	1.34699	6.0	2.7	3.3	1.4	45.0	55.0

until 50 days. The percentage of albumins, however, shows at first an increase and then a decrease, in opposition to the concentration of globulins. During this period of alteration in the proportion of globulin to albumin in the serum, the value for total proteins of the serum does not show any corresponding fluctuation, but is rising continuously. It is known that young rats do not take any large quantity of milk after 23 days of age, but probably solid food only. Wells (1913) found that milk-fed animals show

an increase in the relative amount of the globulins over the control series fed on a mixed diet. The alteration shown in the period between 22 and 30 days may therefore be due to the fact that the rat becomes accustomed to solid food so that the composition of the blood is slowly restored, and the ratios which the various proteins bore to the total protein before the change in diet reappear.

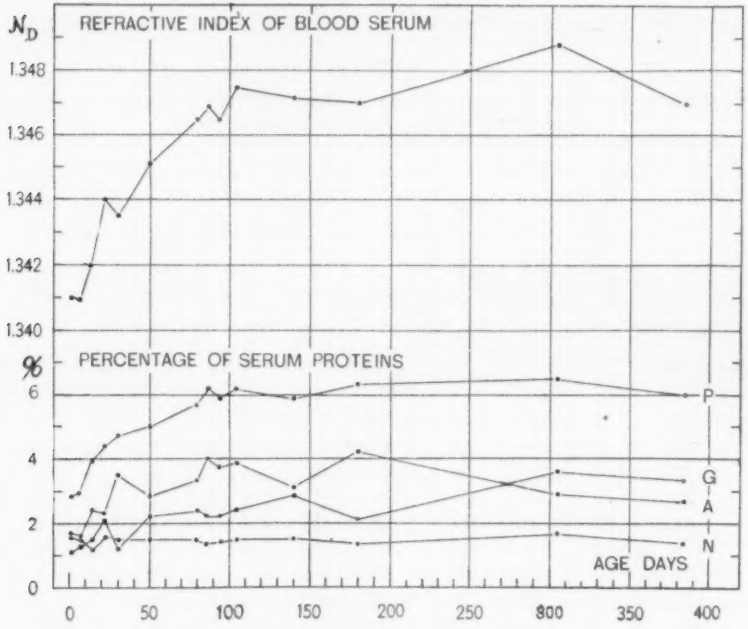

CHART 1. Changes in the refractive index and in the relative amount of serum proteins, based on the data given in Table I.

P = protein. G = globulin.
A = albumin. N = non-protein.

After adjustment to the change in diet the percentage of globulins and the percentage of albumins show a gradual increase with age from 50 to about 90 days. From puberty on the subsequent alteration in the percentage of total proteins is small, though it continues to rise throughout the rest of life. Between 80 to 180 days of age the relative proportion of globulins to albumins shows some irregularity, probably due to high individual variations.

Despite the fact of the irregularities just mentioned there is strong indication that the relative amount of albumin gradually decreases, while that of globulin gradually increases throughout the rest of life so far as examined. We thus find that at the age of about 275 days the ratio of globulin to albumin becomes the reverse of that found at an earlier age.

It should be stated that we always made careful postmortem examinations, and the blood of only those animals which were free from lung infection, as well as any other noticeable pathological alterations, was used for the purpose of analysis.

We infer therefore that the changes in the relative proportion of globulin and of albumin noted in the older rats were not produced by the immediate pathological alterations nor any disease present at the time of killing. It is, however, conceivable that the animals might during their earlier life have suffered from infection, yet so long as we have no direct evidence that the older rats suffered from infectious diseases, I feel justified in making the tentative conclusion that the globulin fraction relative to the albumin fraction shows some increase with advancing age. The percentage of non-protein substances (N) shows no variation corresponding with the age of the rat.

From his observations on rabbits Wells (1913) concludes that the percentage of total proteins increases with age between 21 to 140 days, but that fully adult animals have a slightly lower content.

My observations on the rat, however, show no such tendency of decrease in the content of proteins, even with rats which are 300 days old, although the rats at 385 days do show a slight falling off. Hatai (1918) questions whether or not the adult rabbits used by Wells were entirely normal, and so far as my own data are concerned the rats which were free from visible disease show no indication of falling off, when fully adult and even older. On the contrary, there is a strong tendency for steady increase of the proteins. Wells' data show no decisive increase of the relative amount of globulins at even 1 year of age, while the rats 300 days old give a greater relative amount of globulin than of albumin, thus reversing the relation present at younger periods. I am unable to state whether the serum of rabbits older than 1 year will show similar alterations in the serum proteins as are noted here in the rat.

CONCLUSIONS.

1. The present observation supports the view held by Hatai (1918) that there are in the albino rats three distinct phases in the growth curve of the refractive index of serum; namely, suckling, puberty, and maturity.

2. The percentage of total proteins increases very rapidly during the suckling period, but during the puberty phase growth is slow. The percentage of proteins during the adult period shows only slight increase, but at the age of 385 days a small fall was noticed; this may or may not be significant.

3. The percentage of albumin, though irregular, shows rapid increase for the first 30 days of age. This increase is followed by a rapid fall which soon is followed by a steady rise until the end of sexual maturity. The albumin content at the adult period shows first slow and later a steady relative diminution. The globulin fraction also shows a rapid increase until the end of the suckling period. This rise is followed by a sharp fall and reaches a minimum at the age of 30 days, caused probably by the change of diet from milk to solid food. The relative amount of globulin then shows a steady increase, and at the age of about 275 days the relative amount of globulins becomes greater than that of the albumin.

4. The percentage of the non-protein bodies is practically constant throughout the entire span of life.

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STUDIES OF ACIDOSIS.

XIII. A METHOD FOR TITRATING THE BICARBONATE CONTENT OF THE PLASMA.

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For determination of carbonates and bicarbonates in the absence of proteins and other buffers methods of two general types are available: (1) measurement of the carbon dioxide evolved when the carbonates are decomposed with acid, and (2) titration of the basic part of the carbonate molecule, either by adding excess acid, boiling off the CO_2, and titrating back, or by using an indicator which, like methyl orange, is not sensitive to the weak acidity of carbonic acid. Hitherto only methods of the first type, based on carbon dioxide determination, have proven accurate and practicable for blood analysis.

In order to titrate accurately the bicarbonate of the blood plasma, it is both theoretically and actually necessary, as a net result of the operation, to transform the bicarbonate into the salt of whatever acid is used in the titration, without altering the normal hydrogen ion concentration of the plasma. If the hydrogen ion concentration is altered, the proteins of the plasma bind as a result either acid or alkali in amounts different from those which they bind *in vivo*. Consequently, under such conditions the proteins act either to increase the acid added in titration to an amount more than equivalent to the bicarbonate, or to depress it below the bicarbonate equivalent.

So far as we are aware, no method for titration of the blood plasma with indicators has yet been proposed which meets the following three requirements, all of which are necessary in order to permit interpretation of the results in terms of plasma bicarbonate: (1) The use of the hydrogen ion concentration of nor-

mal blood as the end-point; (2) Removal of the carbon dioxide set free by the added acid; (3) Use of an indicator not affected by the plasma proteins.

It appeared probable that if these requirements could be met, a simple titration of the plasma bicarbonate might be possible, and recent investigations have provided the theoretically necessary data. The work of Lundsgaard (1912), who first demonstrated the constancy of the hydrogen ion concentration of the blood, of Hasselbalch (1911, 1913, 1915), and of a number of subsequent investigators (Michaelis, 1914; Peabody, 1914; Milroy, 1914; Parsons, 1917; Sonne and Jarlφv, 1918), has shown that the pH of human blood under nearly all conditions, normal and pathological, even in acidosis, is approximately constant at 7.4. McClendon has shown that when excess acid is added to the blood plasma, the CO_2 driven off by a stream of hydrogen, and alkali added until the normal pH, as determined by the gas chain, is restored, the acid consumption is about what would be expected from the average bicarbonate content established by Van Slyke, Stillman, and Cullen (1917). An indicator capable of showing pH 7.4 accurately, in the presence of the plasma proteins, has been found in neutral red by Miss Annie Homer (1917). The elimination of carbon dioxide is merely a matter of efficient aeration. Consequently the difficulties to the titration appear to have become theoretically surmountable, and we have found in fact that the simple titration technique described below gives results for plasma bicarbonate identical with those yielded by determining the carbon dioxide.

Description of Method.

In drawing and centrifugating the blood the precautions outlined by Van Slyke and Cullen (1917), for preventing loss or accumulation of carbon dioxide and consequent change in the distribution of bicarbonate between corpuscles and plasma, are to be observed. Oxalate plasma is used. Up to the beginning of the analysis, the blood and plasma are handled exactly as described for the carbon dioxide method.

For the analysis 2 cc. of plasma are pipetted into a round-bottomed flask of 150 to 200 cc. capacity, and 5 cc. of 0.02 N hydrochloric acid are added (this is about 2 cc. of 0.02 N acid in

excess of the bicarbonate normally present). In order to remove the carbon dioxide set free by the acid, the flask is shaken vigorously with a rotary motion, so that the solution is whirled in a thin layer about the inner wall. 1 minute of this treatment is sufficient to remove the carbonic acid so completely that not enough is left to affect the results measurably. The solution is now poured as completely as possible into a 50 cc. Erlenmeyer flask and the walls of the larger flask are rinsed with 20 cc. of water. The water is measured within 1 cc. in a cylinder, and approximately a third is used for each of three washings.

When the solution, measuring about 26 cc., has been transferred to the 50 cc. Erlenmeyer flask, 0.3 cc. of a 0.1 per cent solution of neutral red (dissolved in 50 per cent alcohol) is added. 0.02 N *carbonate-free* NaOH is then run in from a burette (preferably but not necessarily a "micro-burette") until the color of the solution matches that of 29 cc. of a standard phosphate solution, of pH 7.4, contained in a similar 50 cc. flask.[1]

In place of neutral red, 0.3 cc. of a 0.04 per cent solution of phenolsulfonephthalein may be used as indicator, and gives an

[1] The standard solutions of pH 7.2 and 7.4 may be made as follows (Clark and Lubs, 1917), M/20 solutions being obtained. The M/5 KH_2PO_4 contains 27.23 gm. of KH_2PO_4 per liter.

pH	M/5 K H_2PO_4	N/5 NaOH	
	cc.	*cc.*	
7.2	50	35.0	Dilute to 200 cc.
7.4	50	39.5	" " 200 "

They may also be made by Sörensen's method (Sörensen, 1912) from KH_2PO_4 and Na_2HPO_4 as follows, the phosphate concentration being M/15. Both salts prepared especially for this purpose may now be obtained from Merck and Co.

pH	Na_2HPO_4	KH_2PO_4	
	gm.	*gm.*	
7.2	6.89	2.47	Dilute to 1 liter.
7.4	7.72	1.67	" " 1 "

If crystalline $Na_2HPO_4.2H_2O$ is used instead of the anhydrous Na_2HPO_4, the amounts are increased to 8.66 and 9.67 gm. for the solutions of pH 7.2 and 7.4 respectively.

The plasma solution becomes somewhat turbid, but the turbidity partially clears up as the end-point is approached, and the latter can be determined within one drop of the 0.02 N NaOH.

end-point slightly more easy to distinguish than that of neutral red. When phenolsulfonephthalein is used, however, the standard solution must be of pH 7.2 instead of 7.4. The reason for this is that, as pointed out by Miss Homer (1917), when phenolsulfonephthalein is added to a solution containing serum proteins, the pH is considerably higher than that estimated by colorimetric comparison with standard phosphate solutions. The indicator suffers from a protein error. Under the conditions of the analysis here described, however, the error is quite constant at 0.2 to 0.3 pH; *i.e.*, if the color matches that of a standard solution of pH 7.4, the actual pH in the solution under examination is about 7.7 (see Table II). If a standard solution of pH 7.2 is used, however, the titration runs to the same point obtained with neutral red, and, as stated above, the end-point is, to most eyes, somewhat sharper than with neutral red.

For the titrations we have used a 3 cc. micro-burette of the model devised for use with the blood sugar method of Bang. It was provided with an automatic filling device and divided into 0.02 cc. divisions which were over 1 mm. apart, so that readings accurate to within 0.01 cc. could be made easily. The tip was drawn out to a fine point, so that the drops were small, measuring only 0.03 cc. each. Such a burette is extremely desirable, but not absolutely necessary. Even an ordinary 50 cc. burette divided into 0.1 cc. divisions may be used, an error of 0.1 cc. altering the result by only one-thirtieth of the normal, since the bicarbonate in 2 cc. of plasma normally neutralizes about 3 cc. of 0.02 N acid. The accuracy of the method, however, justifies the use of a finer burette, for our results seldom varied from the calculated values, or from agreement with duplicates, by more than 0.05 cc. and were usually within one drop, or 0.03 cc.

The End-Point.—With both indicators, a peculiar phenomenon occurs as the end-point is approached. Each drop appears to change the color past the end-point, but within a few seconds the color shifts back, and it is seen that at least another drop is needed before the genuine end-point is reached. Consequently, the final color comparison should not be made until at least 30 seconds after the last drop of 0.02 N NaOH has been added. Because of this behavior, as well as the character of the color change, it is well, particularly with neutral red, to overrun the end-point by a drop, rather than stop short of it when in doubt.

Calculation of Results.—The number of cc. of 0.02 N NaOH used in the titration is subtracted from the number required to neutralize to the same indicator 5 cc. of the 0.02 N HCl used. This number is, of course, approximately 5, but it usually varies slightly from that because of difference in the factors of acid and alkali, and because of the calibration error of the 5 cc. pipette used for measuring the acid. Consequently the maximum accuracy is obtained by performing a preliminary titration on 5 cc. of the acid plus 20 cc. of the distilled water, using the same pipette, indicator, and end-point as in the plasma titration.

The following is a typical calculation on a normal human plasma.

0.02 N NaOH = HCl added	5.09 cc.
0.02 N NaOH taken in titration	2.03 "
0.02 M $NaHCO_3$ in 2 cc. plasma or 0.01 M $NaHCO_3$ in 1 cc. plasma	3.06 "

3.06 ÷ 100 = 0.0306 = molecular concentration of $NaHCO_3$ in plasma.

3.06 × 22.4 = 68.5 volume per cent CO_2 bound as bicarbonate in the plasma.

Since the titration result represents cc. of 0.01 M $NaHCO_3$ per cc. of plasma, it is transformed into terms of molecular concentration of $NaHCO_3$ in the plasma merely by dividing by 100.

For the sake of comparison with results of bicarbonate determination by the CO_2 method (Van Slyke and Cullen), the molecular concentration is multiplied by 2,240, in order to give results in terms of cc. of CO_2 per 100 cc. of plasma. According to the gas laws the amount of CO_2 contained in a M carbonate solution is 22,400 cc. per liter (measured as CO_2 gas at 0°, 760 mm.) or 2,240 cc. of gas per 100 cc. of solution. Hence multiplying the bicarbonate molecular concentration by 2,240, or multiplying the cc. of 0.02 N acid used in the titration by 22.4, gives the volume per cent of bicarbonate CO_2 in the plasma.

Inversely, of course, dividing the volume per cent of CO_2, as determined by Van Slyke and Cullen, by 2,240 transforms the CO_2 figures into terms of molecular concentration.

The Standard 0.02 N Sodium Hydroxide.—The 0.02 N NaOH as a basis for the determination must, in order to maintain its value, be protected from contact with atmospheric carbon dioxide and from glass. Even standing over night in a burette of soft glass is

likely to result in the solution of enough alkali to raise the titration value of the standard solution. The standard alkali should be kept in paraffined bottles, and the burette filled with fresh solution each day that it is used.

In order to obtain a carbonate-free alkali solution, we use the well known expedient of first dissolving the NaOH in an equal weight of water. Sodium carbonate is insoluble in such a concentrated alkali solution and settles to the bottom. 5.5 cc. of the clear supernatant solution diluted to 5 liters yields an approximately 0.02 N solution which is standardized by titration with neutral red against 0.02 N HCl. In performing the titration it is preferable to run the acid into the alkali, thus titrating from the yellow alkaline color to the acid red. The color change in this direction occurs without the time lag observed when alkali is added to acid.

EXPERIMENTAL.

Determinations on Known Carbonate Solutions.—Solutions of Merck's C.P. Na_2CO_3 were made in concentrations ranging from the carbonate content of normal plasma downwards, and titrated as described for plasma. The results are given in Table I.

Accuracy of End-Point in Plasma Titrations.—Table II shows the results of a number of titrations. After the titrations were finished the pH values of the solution were checked by means of the gas chain. It is seen that the desired value of pH 7.4 was closely approximated as the end-point in each titration, when neutral red was used as indicator with a standard of pH 7.4 for comparison, or when phenolsulfonephthalein was used as indicator with a standard of pH 7.2 for comparison. With the latter indicator, however, and a color standard of pH 7.4, the titration, due to the protein error pointed out by Miss Homer (1917), went to a pH of 7.7. Even this change altered the results by only about 0.001 M, and when a color standard of pH 7.2 was used with phenolsulfonephthalein, the error was completely corrected.

Time Required for Removal of Carbon Dioxide by Aeration.—A series of determinations was performed with portions of the same horse serum, exactly as outlined above under "Description of Method," except that the aeration period was varied. The results in Table III show that an aeration period of 1 minute is

TABLE I.

Na_2CO_3 per liter by weight.*	Concentration of carbonate by methyl orange titration.		Concentration of carbonate by neutral red titration with technique used for blood plasma.	
	0.02 N HCl = 20 cc. carbonate solution.	Concentration calculated as $NaHCO_3$.	0.02 N HCl = 2 cc. solution.	Concentration calculated as $NaHCO_3$.
gm.	*cc.*	*molecular*	*cc.*	*molecular*
1.590	29.1	0.0291	2.90	0.0290
1.090	19.7	0.0197	1.95	0.0195
0.727	13.1	0.0131	1.29	0.0129
0.363	6.57	0.0066	0.67	0.0067

* The carbonate was not dried, hence the actual concentration indicated by the methyl orange titration is only 97 to 98 per cent that indicated by the weight of carbonate taken.

TABLE II.

Serum.	0.02 N NaOH = HCl taken.	0.02 N NaOH used in back titration.	0.02 N NaOH = $NaHCO_3$ in 2 cc. plasma.	Concentration of $NaHCO_3$.	Indicator used.	pH of standard phosphate solution used for end-point comparison.	pH of plasma solution at end of titration.
	cc.	*cc.*	*cc.*	*molecular*			
Horse 1.	5.13	2.41	2.72	0.0272	Neutral red.	7.4	7.36
	5.13	2.44	2.69	0.0269	" "	7.4	7.41
	5.12	2.56	2.56	0.0256	Phenolsulfonephthalein.	7.4	7.72
	5.12	2.53	2.59	0.0259	"	7.4	7.71
	5.12	2.43	2.69	0.0269	"	7.2	7.45
Horse 2.	5.35	2.60	2.75	0.0275	Neutral red.	7.4	7.43
	5.35	2.63	2.72	0.0272	Phenolsulfonephthalein.	7.2	7.45

TABLE III.

Duration of aeration.	Bicarbonate concentration found by titration.
min.	*molecular*
0	0.0117
0.25	0.0220
0.5	0.0255
1.0	0.0260
2.0	0.0260
3.0	0.0260

sufficient, but that 30 seconds is probably not quite enough. Incidentally the result of the titration performed without aeration shows the impossibility of a correct titration without removal of carbon dioxide.

Parallel Determinations of Plasma Bicarbonate by Titration and by Carbon Dioxide Capacity.—The results in Table IV were obtained on the plasmas of blood samples drawn from the ear veins of rabbits and the arm veins of men. The carbon dioxide capacities were determined by one of us, while the titrations were being performed by another.

Parallel Determinations by Titration and Carbon Dioxide Capacity Methods on Normal Human Plasma plus Known Amounts of Acetic

TABLE IV.

Plasma.	CO_2 bound as bicarbonate by 100 cc. of plasma.	Bicarbonate concentration calculated from $CO_2\left(=\frac{CO_2}{2,240}\right)$.	Bicarbonate concentration determined by titration.
	cc.	*molecular*	*molecular*
Normal Rabbit 61	56.7	0.0253	0.0270
" " 62	66.9	0.0298	0.0305
" " 63	46.2	0.0206	0.0199
Diabetic. No acidosis	58.2	0.0259	0.0240
Normal man	61.6	0.0275	0.0265

Acid.—Acetic acid has practically the same dissociation constant (1.8×10^{-5}) as β-hydroxylbutyric (2.0×10^{-5}), and the effect on acid-base balance produced by adding it to plasma is therefore practically identical with the effect of β-hydroxylbutyric acid.

The blood was drawn from one of the authors. 5 cc. portions were treated with water, or water plus 0.1 N acetic acid, making the total volume up to 6.5 cc. Portions were then taken for titration and for carbon dioxide capacity determination (Van Slyke and Cullen method), as described in the preceeding paragraph. Table V gives the results. The CO_2 results are reduced to terms of molecular concentration by dividing the volume per cent CO_2 by 2,240, the theoretical gas constant, as described on page 171. The results are plotted in Fig. 1.

Discussion of the Titration and Carbon Dioxide Capacity Methods for Determination of Plasma Bicarbonate.

It is evident from Fig. 1 that the titration gives theoretically correct values over the entire range of bicarbonate concentrations from normal to zero. The CO_2 determinations agree both with the titration results and with the calculated values for a range of the latter from normal down to somewhat below 0.01 M bicarbonate (22.4 per cent CO_2 capacity). When the bicarbonate falls as low as 0.003 M, however, (6.7 per cent CO_2, or about one-tenth the normal), the CO_2 method gives higher bicar-

TABLE V.

Plasma.	0.1 N acetic acid.	Water.	CO_2 bound as bicarbonate per 100 cc. of treated plasma.	Concentration of bicarbonate.		Fall in concentration.		Concentration of acetic acid added.
				By CO_2 capacity.	By titration.	By CO_2 capacity.	By titration.	
cc.	cc.	cc.	cc.	*molecular*	*molecular*	*molecular*	*molecular*	*molecular*
5	0	1.5	58.9	0.0267	0.0264	0	0	0
5	0.478	1.0	41.6	0.0186	0.0192	0.0081	0.0072	0.0073
5	1.036	0.5	24.2	0.0108	0.0106	0.0155	0.0158	0.0159
5	1.514	0.0	12.8	0.0057	0.0028	0.0205	0.0236	0.0233
5	1.036 of 0.2 N	0.5	0.0	0.0000	−0.0050	—	0.0314	0.0319

bonate than is actually present. This fact was demonstrated and discussed in the original paper on the carbon dioxide capacity method (Van Slyke and Cullen, pp. 327, 328) in the following words:

"Until acid equivalent to about half the plasma bicarbonate has been added the fall in bicarbonate approximately equals in molecular equivalents the amount of acid added. As the amount of acid becomes greater, however, the drop in plasma bicarbonate begins to fall short of the added acid. This is due to the fact that the H_2CO_3 concentration is kept constant, instead of being reduced in proportion to the bicarbonate. The condition is similar to that of the blood in uncompensated acidosis. The $\frac{H_2CO_3}{NaHCO_3}$ ratio, and consequently the C_H is increased. As a result the other plasma buffers (chiefly proteins) bind a measurably greater amount of acid than they could at normal C_H, and the acid so bound is prevented from decomposing bicarbonate.

"The effect on the routine plasma determination is that the bicarbonate determined by our [CO_2] technique denotes a fall in the more severe stages of acidosis which is not quite so great as the actual drop in bicarbonate *in vivo*. The relationship between added acid and decrease in bicarbonate, however, is made so constant by saturating the plasma at a definite carbon dioxide tension that the lack of absolute numerical proportionality in the lower ranges is no practical detriment to the interpretation of results."

In some types of experimental work the theoretical perfection of the titration will doubtless give it an advantage, but for ordi-

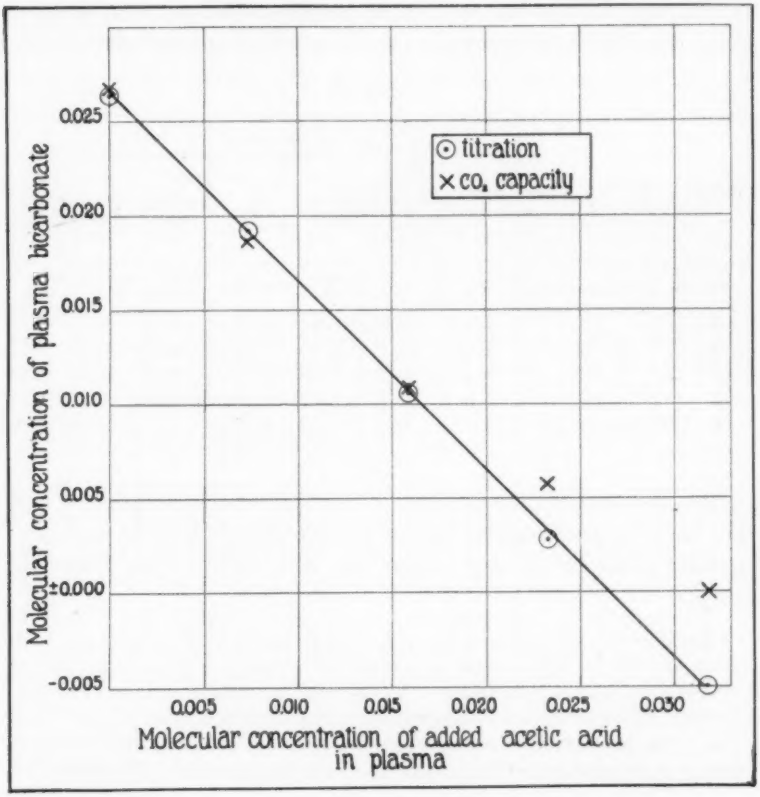

FIG. 1. Effect of addition of acetic acid on bicarbonate concentration of normal human plasma. The straight line represents the bicarbonate concentration calculated on the assumption that the fall in bicarbonate is equivalent to the amount of acid added to the plasma. The results of titrations are represented by ⊙, the results of CO_2 determinations by +.

nary purposes, especially for clinical diagnosis, the choice between the two methods appears entirely one of convenience. The carbon dioxide capacity has the advantage that the extraction and measurement of the gas require slightly less time than even the few minutes necessary for a titration, and require no accurate standard solutions; while the titration has the advantage that it requires no special apparatus. Within the bicarbonate range ordinarily encountered even in acidosis, (Stillman, Van Slyke, Cullen, and Fitz, 1917), the results of either method apparently may be interpreted in terms of the other, so closely do the two agree, and each method is capable of accuracy to within 1 or 2 per cent of the amount of bicarbonate normally present.

CONCLUSION.

The bicarbonate content of serum or oxalate plasma is determined by adding an excess of standard acid (5 cc. of 0.02 N HCl to 2 cc. of plasma), removing the carbon dioxide by rotating the solution for 1 minute about the wall of a flask, and titrating back with 0.02 N NaOH to the original hydrogen ion concentration of blood (pH 7.4) with neutral red as indicator.

The results agree closely with those of the carbon dioxide capacity method over the range of bicarbonate concentrations (0.03 to 0.01 M) ordinarily encountered in man, even in severe acidosis. Below this range the titration continues to give accurate results, while the CO_2 capacity method gives, for reasons discussed in the original paper on the CO_2 method, somewhat higher values. For clinical and most experimental purposes, however, it appears that the two methods give so nearly identical results that they may be used interchangeably.

Micro-titration.—When it is desirable to employ only small amounts of plasma, 0.400 cc. may be transferred to a test-tube of about 20 mm. diameter, and 1.000 cc. of 0.02 N HCl added. The tube is laid in a nearly horizontal position, so that the layer of liquid extends for about 10 cm. along the lower side. The tube is then rotated or rolled back and forth vigorously, but not so roughly as to cause foaming, for not less than 1.5 minutes, in order to cause the carbon dioxide to escape. Three drops of indicator are then added, and the solution is titrated in the test-tube with 0.004 N NaOH to a pH of 7.4, using as a color standard a like volume of phosphate solution in a similar tube.

The 0.004 N NaOH is so rapidly altered by contact with either glass or atmospheric carbon dioxide that it is advisable to make it fresh for each series of analyses by diluting 10 cc. of 0.1 N NaOH to 250 cc. with distilled water that has been freed of CO_2 either by boiling or by shaking in an evacuated flask. The control titration of the 0.004 N NaOH against the 1 cc. of 0.02 N HCl should be performed immediately before the plasma titration.

The calculation is the same as in the larger titration. The number of cc. of 0.004 N NaOH used is subtracted from the number of cc. (approximately 5) required to neutralize the HCl in the control titration. The difference divided by 100 represents the molecular concentration of bicarbonate in the plasma, while the difference multiplied by 22.4 indicates the volume per cent of bicarbonate CO_2.

With care in the calibration of pipettes, and especially in the control of the 0.004 N NaOH, results nearly and perhaps quite as accurate as in the larger titration appear attainable.

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THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE LIQUEFACTION OF GELATIN.

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Fischer and Coffman[1] show that "there is a progressive increase in the tendency of gelatin to go into solution in mixtures of the salts of polybasic acids as the amount of acid or alkali in these mixtures is increased from a given low point.[2] In view of the well known relation of hydrogen ion concentration to various equilibria, it seemed desirable to investigate its effect in the liquefaction of gelatin since the results of Fischer and Coffman do not cover this point directly. Also it seemed desirable that the results of Fischer and Coffman be checked, as they have an interest wider than the physiological application. The present paper is intentionally limited to a presentation of chemical and physical data, and does not discuss their medical applications.

According to the scheme of Fischer and Coffman, solutions were made up from known volumes of standardized acids, salts, and alkalies so that a graded variation from acid to alkali through neutral solution was secured, gelatin solution of definite strength being added in each case and the whole made up to a given volume (10 cc.) with conductivity water. Thus the gelatin was always contained in the same volume of water. The pH values in gelatin solutions were determined by the customary method of adding a few drops of indicator solution and comparing the color just produced with the color of the same quantity of the same indicator in standard solutions of known hydrogen ion concentration (checked electrometrically). A blank test of each solution was

[1] Fischer, M. H., and Coffman, W. D., *J. Am. Chem. Soc.*, 1918, xl, 304.

[2] The isoelectric point for gelatin is given as pH 4.60 by Michaelis and Grineff, cited by Walpole, G. S., *Biochem. J.*, 1914, viii, 628.

made colorimetrically for the pH[3] value in absence of gelatin; also, a test on the pH of the solution of gelatin alone in conductivity water.

Table I shows the composition and pH of phosphoric acid (approximately 0.01 N) through phosphates to sodium hydroxide in twenty-one stages, the higher concentration in alkali not being measured colorimetrically owing to lack of a suitable indicator, and for our present purpose being so far from the liquefaction pH of gelatin as not to warrant the extra work of electrometric measurement at this time. In Table I is also given the effect of the solutions cited upon the physical state of gelatin (3 per cent) at 21°C., together with the pH of each gelatin-phosphate system as determined by indicators.[4] The data in Table I are shown graphically in Fig. 1, where the number of the solution is indicated on the ordinate (from Nos. 2 to 21, passing from acid to alkali) and the observed pH, shown by the indicator, as on the abscissa. Curve 1 represents the solutions alone; Curve 2 shows the effect of the 3 per cent gelatin. The amphoteric effect of the gelatin is clearly in evidence, the hydrogen ion concentration being depressed in acid solution and raised in alkaline solution.

The gelatin itself in water showed acid reaction to the extent of pH = 5.6. The gelatin was the best obtainable for culture work, and was redissolved, filtered, and precipitated from 95 per cent alcohol twice, and dried at 105°C. for some hours. Between pH 8.4 and pH 9.2 the gelatin begins to liquefy and is not completely fluid at pH 9.6.

Table II shows the same effect of solutions upon gelatin using solutions approximately 0.1 N ranging from H_3PO_4 to NaOH. Fig. 2 is plotted in the same manner as Fig. 1, Curve 1 being again for the solutions alone, while Curve 2 shows the effect of adding 3 per cent of gelatin. In this series also the gelatin shows a marked

[3] $pH = \log \frac{1}{\text{concentration of hydrogen ions}}$ thus, a solution 0.01 normal with regard to hydrogen ions would have a pH = 2.

[4] The authors are aware that a protein effect may be exercised upon the indicator by the gelatin, but this effect appears not to mask the pH determination for the present purposes. Most of the indicators are from the series of Clark and Lubs, (*J. Bacteriol.*, 1917, ii, 1) who chose them in part because of their relatively small errors in the presence of protein.

amphoteric effect, being acid in the alkali region and basic in the acid region.

This same amphoteric action is shown, using citric acid with 2 per cent gelatin and 3 per cent gelatin, in Tables III and IV and Fig. 3. In sodium bicarbonate solutions, Table V and Fig. 4, the gelatin always in alkaline solution displaces the pH toward the acid region. Thus, the action of gelatin in these buffer solutions tends to displace their pH value. The amphoteric action of gelatin in these solutions is a real effect.

From the data given in the tables, graphical representations of the effect of pH upon the liquefaction of gelatin have been secured by using an arbitrary ordinate scale of solidification; 100 being a set gel and 0 the mobile liquid, the semisolid condition is taken as 50. The abscissæ represent pH.

Fig. 5 shows that a 3 per cent gelatin liquefies between 8.4 and 9.2 in sodium bicarbonate solutions; between 8.4 and 10.1 in 0.01 N phosphoric acid solutions; and between 8.4 and 9.6 for citric acid solutions. The data are given in Tables I, IV, and V. Fig. 6, plotted from Tables III and IV shows the pH zone throughout which the gelatin remains solid in citrate solutions. As would be expected the stronger gelatin solution is solid over a wider pH range.

Fig. 7 shows the effect of temperature upon the setting of 3 per cent of gelatin in 0.1 N phosphoric acid-sodium hydroxide solutions from 18–25°C.; the narrowing of the pH range for solid gelatin solutions due to rise in temperature is clearly seen.

Throughout all this preliminary work the criterion of solidity in a set gelatin is rough, being simply that it must show a jelly-like elasticity.

CONCLUSIONS.

1. From the above data it is clear that the setting of gelatin is influenced by the hydrogen ion concentration of the medium and unless the gelatin is destroyed this effect is probably reversible.

2. Gelatin in the concentrations used is not without effect upon the buffer solutions, displacing the pH in such a manner as one would expect from an aggregate of amino-acids acting amphoterically.

TABLE I.

Setting of Gelatin Solutions (3 Per Cent) with Phosphoric Acid through Phosphates to Sodium Hydroxide. Approximately 0.01 Normal Alkali.

Solution No.	Phosphate solution plus 5 cc. 6 per cent gelatin solution plus water to 10 cc. volume.								Phosphate solution plus water to 10 cc. without gelatin.	
	N H_3PO_4	M NaH_2PO_4	M Na_2HPO_4	M Na_3PO_4	N NaOH	pH	Indicator used.	Physical state 21°C.	pH	Indicator used.
	cc.	cc.	cc.	cc.	cc.					
1						5.6	M.R.	Solid.	7.2	P.R.
2	0.1					4.6	B.P.B.	"	2.3	T.B.
3	0.08	0.02				4.8	"	"	2.5	"
4	0.06	0.04				5.0	M.R.	"	3.2	B.P.B.
5	0.04	0.06				5.2	"	"	3.4	"
6	0.02	0.08				5.5	"	"	4.6	M.R.
7		0.10				5.7	"	"	6.0	B.C.P.
8		0.08	0.02			6.0	B.C.P.	"	6.7	"
9		0.06	0.04			6.3	"	"	7.0	P.R.
10		0.04	0.06			6.5	"	"	7.3	"
11		0.02	0.08			6.7	"	"	8.4+	"
12			0.10			7.1	P.R.	"	9.4	P.P.
13			0.08	0.02		7.5	"	"	10.0+	"
14			0.06	0.04		8.0	"	"		
15			0.04	0.06		8.4	"	"		
16			0.02	0.08		9.2	T.B.	Semisolid.		
17				0.10		9.6	"	"		
18				0.08	0.02	10.+	P.P.	Liquid.		
19				0.06	0.04			"		
20				0.04	0.06			"		
21				0.02	0.08			"		
22					0.10			"		

M.R. = methyl red. P.R. = phenol red.
B.P.B. = bromophenol blue. T.B. = thymol blue.
B.C.P. = bromocresol purple. P.P. = phenolphthalein.

TABLE I. *Setting of Gelatin Solutions (3 Per Cent) with Phosphoric Acid Through Phosphates to Sodium Hydroxide.*

TABLE II

Setting of Gelatin Solutions (3 Per Cent) with Phosphoric Acid Through Phosphates to Sodium Hydroxide.

Solution No.	Phosphate solution plus 5 cc. 6 per cent gelatin solution plus water to 10 cc. volume.							Physical state.				Phosphate solution plus water to 10 cc. without gelatin.	
	N H_3PO_4	M NaH_2PO_4	M Na_2HPO_4	M Na_3PO_4	N NaOH	pH	Indicator used.	18°C.	20.5°C.	22°C.	25°C.	pH	Indicator used.
	cc.	cc.	cc.	cc.	cc.								
1						5.2	M.R.	Solid.	Solid.	Solid.	Solid.	7.2	P.R.
2	1.0					2.8	M.Y.	Liquid.	Liquid.	Liquid.	Liquid.	1.3	T.B.
3	0.8	0.2				3.2	B.P.B.	"	"	"	"	2.1	"
4	0.6	0.4				3.6	"	Semisolid.	Semisolid.	"	"	2.4	"
5	0.4	0.6				4.0	"	Solid.	Solid.	Semisolid.	"	2.8	"
6	0.2	0.8				4.2	M.R.	"	"	"	Semisolid.	3.2	B.P.B.
7		1.0				4.4	"	"	"	Solid.	"	4.4	"
8		0.8	0.2			5.+	"	"	"	"	Solid.	5.8	B.C.P.
9		0.6	0.4					"	"	"	"	6.4	"
10		0.4	0.6					"	"	"	Semisolid.	6.8	"
11		0.2	0.8			6.8	P.R.	"	"	"	"	7.2	P.R.
12			1.0			7.3	"	"	"	Semisolid.	"	8.5	"
13			0.8	0.2		7.9	"	"	Semisolid.	Liquid.	Liquid.		
14			0.6	0.4				Semisolid.	"	"	"	9.6	T.B.
15			0.4	0.6				Liquid.	Liquid.	"	"		P.P.
16			0.2	0.8				"	"	"	"	10+	
17				1.0				"	"	"	"	not measurable with indicators at hand.	
18				0.8	0.2			"	"	"	"		
19				0.6	0.4	8.2	P.R.	"	"	"	"		
20				0.4	0.6	8.4	"	"	"	"	"		
21				0.2	0.8			"	"	"	"		
22					1.0	9.0	P.P.	"	"	"	"		

M.R. = methyl red.
M.Y. = metaniline yellow (*m*-benzolsulfonicdiphenylamine).
B.P.B. = bromophenol blue.
P.R. = phenol red.
P.P. = phenolphthalein.
T.B. = thymol blue.
B.C.P. = bromocresol purple.

TABLE III.

Setting of Gelatin Solutions (2 Per Cent) with Citric Acid through Citrates to Sodium Hydroxide.

Solution No.	Citrate solution plus 5 cc. 4 per cent gelatin solution plus water to 10 cc. volume.								
	N citric acid.	M/4 NaH_2 citrate.	M/4 Na_2H citrate.	M/4 Na citrate.	N NaOH	pH	Indicator used.	Physical state.	
								20°C.	12°C.
	cc.	cc.	cc.	cc.	cc.				
1						5.6	M.R.	Solid.	Solid.
2	1.0					4.3	B.P.B.	Liquid.	Liquid.
3	0.8	0.8				4.4	"	"	
4	0.6	1.6				4.4	"	"	
5	0.4	2.4					"	"	
6	0.2	3.2					"	"	
7		4.0					"	"	
8		3.2	0.8			5.0	"	"	
9		2.4	1.6				"	"	
10		1.6	2.4				"	"	Semisolid.
11		0.8	3.2				"	"	"
12			4.0				"	"	"
13			3.2	0.8			"	Semisolid.	Solid.
14			2.4	1.6		5.0	M.R.	"	"
15			1.6	2.4		5.2	"	Solid.	"
16			0.8	3.2		5.6	"	"	"
17				4.0		6.8	B.C.P.	"	"
18				3.2	0.2	9.0	P.R.	Liquid.*	Liquid.*
19				2.4	0.4	9.6	P.P.	"	
20				1.6	0.6	10.+	"	"	
21				0.8	0.8			"	
22					1.0			"	

* The system was turbid up to Solution 18 at both 20° and 12°, but at this point a clear liquid was obtained with some settled out precipitate. Evidently the gelatin was not of the highest grade.

M.R. = methyl red. B.C.B. = bromocresol blue.
B.P.B. = bromophenol blue. P.R. = phenol red.
P.P. = phenolphthalein.

TABLE IV.

Setting of Gelatin Solutions (3 Per Cent) with Citric Acid through Citrates to Sodium Hydroxide.

Solution No.	Citrate solution plus 5 cc. 6 per cent gelatin plus water to 10 cc. volume.								
	N citric acid.	M/4 NaH_2 citrate.	M/4 Na_2H citrate.	M/4 Na_3 citrate.	N NaOH	pH	Indicator used.	Physical state.	
	cc.	cc.	cc.	cc.	cc.				
1						5.6	M.R.	Solid.	
2	1.0					4.3	B.P.B.	"	2.3*
3	0.8	0.8				4.4	"	"	2.4
4	0.6	1.6				4.4	"	"	2.8
5	0.4	2.4				4.4	"	"	2.8
6	0.2	3.2				4.4	"	"	
7		4.0				4.4	"	"	3.0
8		3.2	0.8			4.8	"	"	
9		2.4	1.6			4.8	"	"	
10		1.6	2.4			4.8	"	"	
11		0.8	3.2			4.8	"	"	
12			4.0			4.8	"	"	
13			3.2	0.8		4.8	"	"	
14			2.4	1.6		5.0	"	"	
15			1.6	2.4		5.2	"	"	
16			0.8	3.2		5.6	"	"	
17				4.0		6.8	"	"	8.2
18				3.2	0.2	9.0	P.R.	Semisolid.	
19				2.4	0.4	9.6	P.P.	Liquid.†	
20				1.6	0.6	10.+	"		
21				0.8	0.8				
22					1.0				

* The numbers indicate pH values of the citrate solutions without gelatin.

† Very sharp change to mobile liquid.

M.R. = methyl red. P.R. = phenol red.

B.P.B. = bromophenol blue. P.P. = phenolphthalein.

TABLE V.

Setting of Gelatin Solutions (3 Per Cent) with Sodium Bicarbonate through Sodium Carbonate to Sodium Hydroxide.

Solution No.	5 cc. 6 per cent gelatin plus solutions tabulated plus water to 10 cc.							
	M $NaHCO_3$	M Na_2CO_3	N NaOH	pH	Indicator used.	Physical state. 22°C.	$NaHCO_3$ solution without gelatin pH.	Indicator used.
	cc.	cc.	cc.					
1				5.6	M.R.	Solid.		
2	0.20			7.5	P.R.	"	8.8	T.B.
3	0.18	0.02		8.4	"	"	9.3	"
4	0.16	0.04		8.6	"	Semisolid.	9.4	"
5	0.14	0.06		8.9	T.B.	"	9.5	"
6	0.12	0.08		9.2	"	Liquid.	9.6	"
7	0.10	0.10		9.4	"	"	10.0	P.P.
8	0.08	0.12		9.6	"	"		
9	0.06	0.14		10.0	P.P.	"		
10	0.04	0.16				"		
11	0.02	0.18				"		
12		0.20				"		
13		0.18	0.02			"		
14		0.16	0.04			"		
15		0.14	0.06			"		
16		0.12	0.08			"		
17		0.10	0.10			"		
18		0.08	0.12			"		
19		0.06	0.14			"		
20		0.04	0.16			"		
21		0.02	0.18			"		
22			0.20			"		

M.R. = methyl red. T.B. = thymol blue.
P.R. = phenol red. P.P. = phenolphthalein.

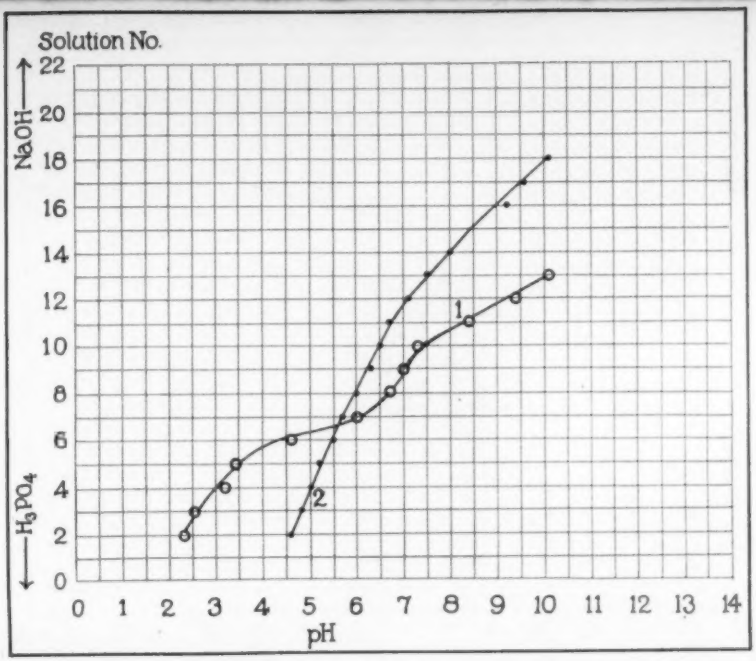

FIG. 1. Amphoteric effect of gelatin. Curve 1, pH of phosphoric acid 0.01 N through phosphates to sodium hydroxide; Curve 2, the same, but with 3 per cent gelatin.

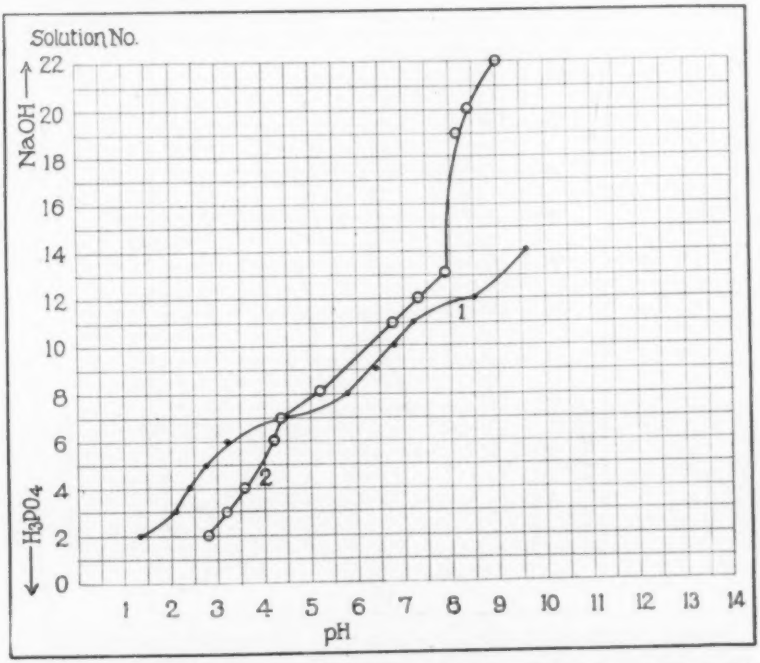

FIG. 2. Amphoteric effect of gelatin. Curve 1, pH of phosphoric acid 0.1 N through phosphates to sodium hydroxide; Curve 2, the same, with 3 per cent gelatin.

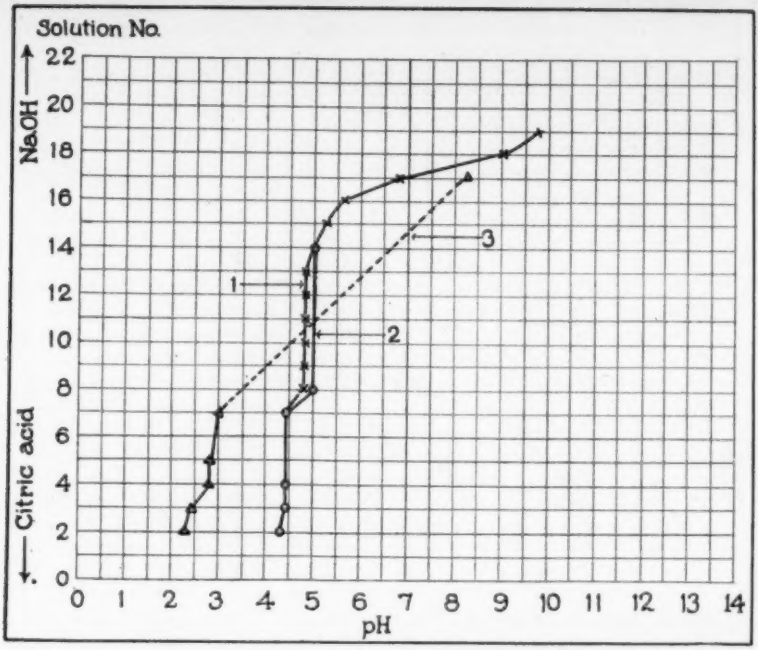

FIG. 3. Amphoteric effect of gelatin. Curve 1, pH of citric acid through citrates to sodium hydroxide with 2 per cent gelatin; Curve 2, the same, but with 3 per cent gelatin; Curve 3, the same, but with no gelatin present.

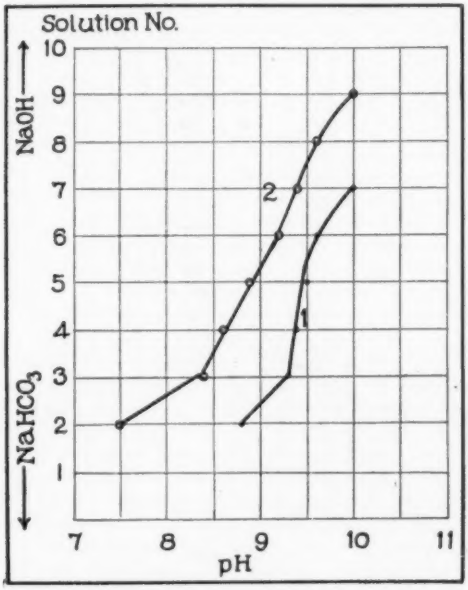

FIG. 4. Acid effect of gelatin. Curve 1, pH of sodium bicarbonate to sodium hydroxide; Curve 2, the same, with 3 per cent gelatin.

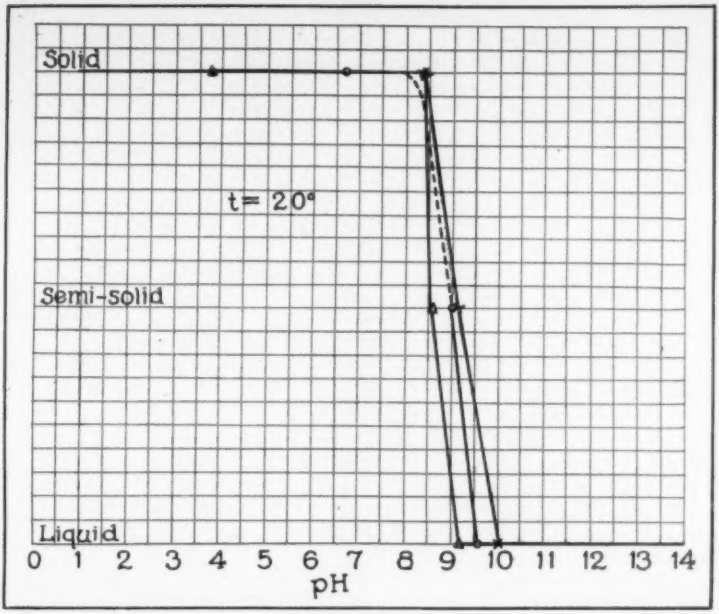

FIG. 5. Comparison of effect of citrate, phosphate, and bicarbonate solutions upon the setting of gelatin (3 per cent) at 20°C. O = citrates, X = phosphates, Δ = bicarbonates.

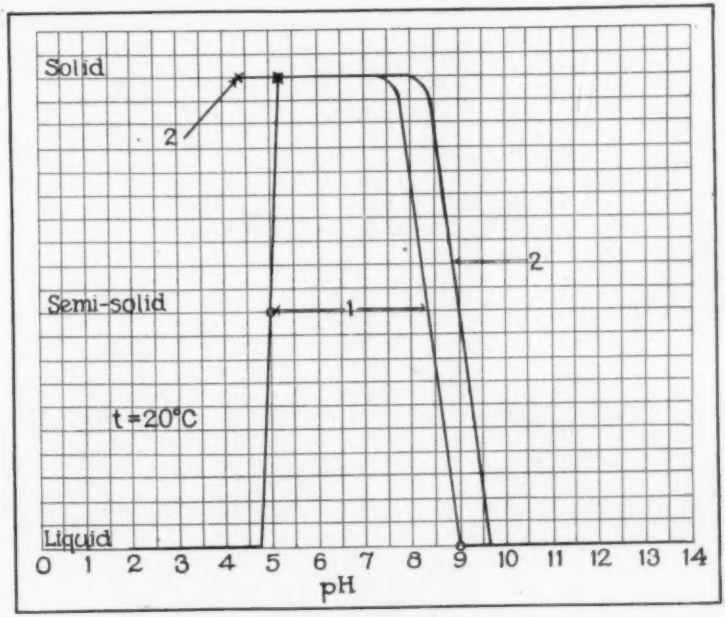

FIG. 6. Effect of pH upon setting of gelatin in citric acid to sodium hydroxide solutions. Curve 1. with 2 per cent gelatin: Curve 2, with 3 per cent gelatin.

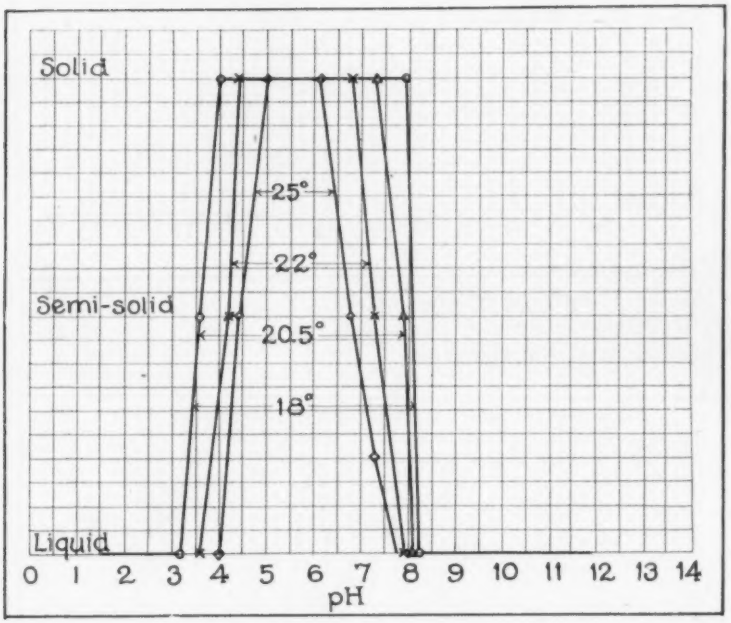

FIG. 7. Effect of temperature upon pH at which setting of gelatin takes place. Range 18–25°C. Medium, phosphoric acid 0.1 N through phosphates to sodium hydroxide (aqueous solution).